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Oxygen Relations of Nitrogen Fixation in Cyanobacteria

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INTRODUCTION

According to fossil records, cyanobacteria have existed on Earth for over 2.5 billion years (287). Ancient cyanobacteria were apparently the first photosynthetic organisms capable of utilizing water as the ultimate source of electrons for the generation of reductant in photosynthesis. The concomitant release of free oxygen was one of the most significant events in the planet's history. It has caused the gradual transformation of the primordial reducing atmosphere to an oxidizing one, thus enabling the development of the aerobic mode of heterotrophic metabolism in the living world. Oxygenic photosynthesis, as evolved in cyanobacteria and evidently inherited by green plants, is the most important mechanism for capturing solar energy on Earth. The chemically bound energy and reductant generated in light reactions are used for carbon dioxide fixation. Oxygenic photosynthesis is the principal force in the cyclic transformation of carbon and oxygen and thus in the maintenance of the critical gaseous composition of the atmosphere.

Many cyanobacteria also contribute greatly to the nitrogen economy of aquatic and terrestrial habitats through their ability to fix atmospheric nitrogen (91). This process of biological nitrogen fixation compensates for the continuous loss of combined nitrogen in the biosphere incurred by the

TABLE 1. Principal groups of cyanobacteria[a]

Section[b]	Basic morphology	Reproduction	Order (family)[c]	Typical genera
I	Unicellular or colonial	Binary fission	*Chroococcales*	*Gloeobacter*, *Synechococcus*, *Synechocystis*, *Microcystis*, *Gloeothece*, *Gloeocapsa*, *Chroococcus*
II	Unicellular or colonial	Budding, multiple fission	*Chamaesiphonales* *Pleurocapsales*	*Chamaesiphon*, *Dermocarpa*, *Dermocarpella* *Xenococcus*, *Myxosarcina*, *Pleurocapsa*
III	Filamentous nondifferentiated	Trichome fragmentation, hormogonia	*Nostocales* (*Oscillatoriaceae*)	*Oscillatoria*, *Microcoleus*, *Spirulina*, *Pseudanabaena*, *Plectonema*, *Lyngbya*, *Phormidium*
IV	Filamentous heterocystous	Trichome fragmentation, hormogonia, akinetes	(*Nostocaceae*) (*Rivulariaceae*) (*Scytonemaceae*)	*Anabaena*, *Aphanizomenon*, *Nostoc*, *Nodularia*, *Anabaenopsis* *Calothrix*, *Gloeotrichia*, *Rivularia* *Scytonema*, *Tolypothrix*
V	Branched filamentous heterocystous	Trichome fragmentation, hormogonia, akinetes	*Stigonematales*	*Fischerella*, *Mastigocladus*, *Stigonema*, *Westiella*

[a] Reprinted with permission from Fay (91a).
[b] From Rippka et al. (270).
[c] From Fritsch (105a).

reverse process of bacterial denitrification. A considerable proportion (5 to 50%) of microbially fixed nitrogen is further lost to the atmosphere in the form of molecular nitrogen as a result of denitrification during biomass burning (187).

The reduction of dinitrogen to ammonia during nitrogen fixation is catalyzed by an enzyme system, nitrogenase, which is extremely oxygen sensitive. Nitrogenase is rapidly and irreversibly inactivated when extracted from cells in the presence of free oxygen (276). The existence in the same organism of two conflicting metabolic systems, oxygen-evolving photosynthesis and oxygen-sensitive nitrogen fixation, appears to be a puzzling paradox. This review will summarize our understanding of the various mechanisms present in cyanobacteria that serve to resolve this incongruity. For readers less familiar with the topic, however, a brief introduction to cyanobacteria, nitrogen fixation, and oxygen sensitivity of nitrogenase is desirable and will precede the main discussion.

THE CYANOBACTERIA

Cyanobacteria, also known as blue-green algae, are a diverse group of gram-negative photosynthetic prokaryotes. They incorporate simple unicellular organisms (with a frequent tendency for aggregation and colony formation), plain unbranched filamentous forms, and strains with a more elaborate branched filamentous structure. In a major revision of cyanobacterial taxonomy, Stanier and colleagues have distinguished five subgroups or sections (Table 1) that more or less, although not in detail, correspond to the earlier orders or families designated as *Chroococcales*, *Chamaesiphonales*, *Pleurocapsales*, *Nostocales* (including the families *Oscillatoriaceae*, *Nostocaceae*, and *Rivulariaceae*), and *Stigonematales* (270).

The basically prokaryotic cellular organization of cyanobacteria is characterized by an often massive presence of intracellular membranes, the thylakoids, which incorporate or hold the photosynthetic pigments and constitute the photosynthetic apparatus of the cell (100). The cytoplasm usually contains a variety of granular inclusions of diverse composition and function. The planktonic forms are distinguished by the presence in their cells of gas vacuoles, which are structures composed of many hundreds of tiny gas-filled vesicles (351). They provide buoyancy to the cells and enable cyanobacteria to occupy an optimum position within the water body.

Many cyanobacteria produce differentiated cells that either serve for protection, perennation, and reproduction or perform a specific vegetative function. Under conditions of nitrogen limitation, many filamentous species differentiate cells called heterocysts, whose sole function is nitrogen fixation. They are formed by the transformation of about every 10th to 15th vegetative cell and are more or less evenly distributed along the filaments (100).

Photosynthetic energy conversion in the cyanobacteria is similar to that in eukaryotic algae and in green plants (157). It involves the operation of two distinct photosystems, photosystem I and photosystem II (PS I and PS II, respectively), which are linked in series and interact through a chain of electron carriers. The major light-harvesting pigments, which contribute to the color of cyanobacteria, are the phycobiliproteins (phycocyanin, allophycocyanin, and phycoerythrin). They are organized as supramolecular complexes, called phycobilisomes, which are attached in regular arrays to the thylakoid membranes (124). Excitation energy is transferred from phycoerythrin through phycocyanin and allophycocyanin to chlorophyll *a* in the reaction centers. Phycobiliproteins may be present at very high concentrations and may also serve as reserve protein. They are water soluble and easily dissociate from the thylakoid membranes.

Some cyanobacteria can use sulfide, molecular hydrogen, and other compounds, instead of water, as electron donors in an anoxygenic type of photosynthesis that is dependent only on the PS I reaction, as in the photosynthetic bacteria (237).

Photoautotrophy, the assimilation of carbon dioxide in light, is the principal and preferred mode of metabolism in cyanobacteria, although some strains are capable of slow chemoheterotrophic growth in the dark or in dim light (299). Exogenous organic substrates can also support nitrogen fixation in the dark. The primary route by which carbon is assimilated in cyanobacteria is the reductive pentose phos-

phate pathway (Calvin-Benson cycle) involving the two key enzymes phosphoribulokinase and ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) (328).

Cyanobacteria were thought to exhibit very low rates of endogenous respiration, but in fact these rates are comparable to those of eukaryotic algae (251). Their respiratory activity, however, shows little response to the presence of external substrates. The main reserve substrate is glycogen, and the route of substrate catabolism in cyanobacteria is the oxidative pentose phosphate pathway. The respiratory electron transport chain is apparently located both in the plasma membrane and within the thylakoid membranes (251). In the latter it may share components with the photosynthetic electron transport system.

Cyanobacteria are common in a great variety of natural habitats and are often abundant in fresh water and seawater, as well as in terrestrial environments (100). A few genera of heterocystous cyanobacteria are engaged in specific symbiotic associations with algae, fungi, liverworts, ferns, and higher plants. In all these associations the endosymbiotic cyanobacteria become extensively modified in their appearance, biochemical properties, and metabolic activities, which results in extremely high rates of nitrogen fixation and in the transfer of the bulk of the fixed nitrogen to the host organism (324).

NITROGEN FIXATION

Although biological nitrogen fixation is limited to prokaryotic microorganisms, the ability to fix nitrogen is by no means a universal property within the group. Even though it is present in the representatives of otherwise unrelated families among gram-positive, gram-negative, photosynthetic, heterotrophic, obligate anaerobic, facultative, and aerobic bacteria, within the families the property may be restricted to only a few genera or species (260). The sporadic occurrence of nitrogen fixation within the prokaryotes seems to suggest that the ability to fix nitrogen might have arisen during an early period of bacterial evolution (31, 51, 205, 311). The corresponding genetic information could have been lost in many strains, possibly in the course of adaptation to changing environmental conditions and the associated selective evolutionary pressures upon microorganisms. An alternative interpretation of the scattered occurrence of nitrogen fixation based on the possibility of horizontal gene transfer (295) recently has been considered less feasible (262), particularly in view of results indicating that nitrogenase genes evolved concurrently with the bacteria that carry them (152).

The concept of a common origin of nitrogen fixation is consistent with the strikingly similar physical and chemical characteristics of the nitrogen-fixing enzyme system present in otherwise dissimilar organisms (81, 201, 218). The enzyme complex consists of two component proteins. One is a Mo-Fe protein, called dinitrogenase, and the other is an Fe-containing protein, dinitrogenase reductase. Dinitrogenase is a tetramer composed of two pairs of different subunits ($2\alpha 2\beta$); it contains four (4Fe-4S) clusters and two molecules of Mo-Fe cofactor. The Mo-Fe cofactor is an essential component of dinitrogenase; it contains eight Fe and six S atoms per Mo atom, without forming 4Fe-4S clusters. The molecular mass of dinitrogenase is about 245 kDa. Dinitrogenase reductase is a dimer composed of two identical subunits with a single (4Fe-4S) cluster and a molecular mass of about 64 kDa (39). The Mo-Fe cofactor is thought to effect the binding and reduction of dinitrogen to ammonia:

$$N_2 + 6H^+ + 6e^- \rightarrow 2NH_3$$

Electrons for the reduction of N_2 are supplied by dinitrogenase reductase. The reaction is highly endergonic, requiring about 12 to 15 mol of ATP per mol of N_2 reduced.

Nitrogenase can reduce a number of other substances, such as acetylene, hydrogen azide, hydrogen cyanide, or nitrous oxide. Molecules of these compounds contain a triple bond, like N_2. Of these, acetylene, which is reduced to ethylene, is particularly important because both acetylene and ethylene can be detected conveniently and with great accuracy by gas chromatography. The acetylene reduction assay is now used routinely to estimate nitrogenase activity.

Nitrogen fixation is always accompanied by a variable amount of proton reduction and H_2 formation, even under an N_2 pressure of 50 atm (5,065 kPa) (25, 297). This nitrogenase-catalyzed production of H_2 represents a loss of energy and reductant, which is partially recovered by the action of an uptake hydrogenase present in many nitrogen-fixing organisms. It catalyzes the oxidation of H_2 in an oxy-hydrogen or Knallgas reaction. The physiological electron donor for nitrogenase in most N_2-fixing organisms is ferredoxin, or flavodoxin under conditions of iron deficiency; the donor in turn receives electrons from reductants generated in intermediary metabolism.

Ammonia, the product of nitrogen fixation, is assimilated mainly via the glutamine synthetase-glutamate synthase pathway, in the same way as exogenously supplied ammonia (211, 337). Other enzymes, such as alanine dehydrogenase and glutamate dehydrogenase, may have a minor role in ammonia incorporation.

All nitrogen-fixing organisms or diazotrophs preferentially assimilate ammonia or other forms of fixed nitrogen (nitrate, urea, amino acids, etc.), and may synthesize nitrogenase only when such sources of combined nitrogen are absent in the medium. In some nitrogen-fixing organisms the supply of ammonia may rapidly inhibit nitrogenase activity. This reversible inhibition by ammonia is attributed to a modification of one subunit of nitrogenase reductase (132, 267). Ammonia in all nitrogen-fixing organisms is primarily involved, through glutamine synthetase, in the regulation, at the transcriptional level, of nitrogenase synthesis (203, 291, 341). Oxygen, which irreversibly inactivates nitrogenase, also represses the synthesis of its component proteins (76, 326). The regulation by ammonia, and by oxygen, of nitrogenase synthesis prevents the wastage of energy and reductant and may have developed as an evolutionary response to the high costs of nitrogen fixation.

Genetic control of nitrogen fixation and the organization of genes coding for the synthesis of the N_2-fixing enzyme complex display basic similarities among N_2-fixing organisms, but show also some variations that may relate to the specific requirements of groups or individual strains (146). The nitrogen fixation genes, or *nif* genes, are usually organized in a cluster within the bacterial chromosome. As many as 17 *nif* genes have been identified in the extensively studied bacterium *Klebsiella pneumoniae*. Three of these genes, *nifH*, *nifD*, and *nifK*, designated as structural *nif* genes, encode the components of the nitrogenase complex; *nifD* and *nifK* code for the α and β subunits of dinitrogenase, respectively, while dinitrogenase reductase is encoded by *nifH*. These genes have been well conserved and detected in all species studied. The grouping of associated regulatory genes, however, shows more variability. Regulatory genes

appear to respond to environmental signals, indicating whether conditions are supportive or unfavorable for nitrogen fixation (131). The genes *nifL* and *nifA* form the operon *nifLA*. Their products regulate the expression of all other *nif* genes (138, 179). The *nifA* gene product is required for their expression (positive control), and the *nifL* gene product prevents their expression (negative control) in the presence of oxygen or combined nitrogen. Expression of the *nifLA* operon, however, requires activation by the products of two other so-called nitrogen regulatory genes, *ntrA* and *ntrC*. It is thought that in the presence of ammonia or oxygen the product of the *nifL* gene inactivates the *nifA* protein (146).

EFFECT OF OXYGEN ON NITROGEN FIXATION

The inhibitory effect of atmospheric oxygen on nitrogen fixation was noted during early studies with free-living nitrogen-fixing organisms as well as with symbiotic systems (14, 23, 143, 210, 245). The finding that nitrogen fixation in obligate anaerobic and facultative bacteria will occur only in the total absence or at very low concentrations of oxygen was attributed to the general intrinsic sensitivity of these organisms to oxygen.

True comprehension of the particular extreme sensitivity of the nitrogen-fixing enzyme system to oxygen has been brought about by a number of significant findings. The first was the long-sought conclusive evidence that nitrogen fixation is essentially a reductive process and that ammonia is the primary product of nitrogen fixation (15, 217). That oxygen interferes with this reaction was therefore no longer surprising. Second was the observation that facultative bacteria, such as *Klebsiella pneumoniae* (181, 250) or *Bacillus polymyxa* (133), and microaerophilic organisms, such as *Mycobacterium flavum* (21), are able to synthesize nitrogenase only under anaerobic or microaerobic conditions, whereas the same bacteria could grow in the presence of air when provided with a suitable source of combined nitrogen (156). Then there was the demonstration that oxygen inhibits nitrogenase activity even in the typically aerobic *Azotobacter* species. Burk (38) showed that the efficiency of nitrogen fixation (N_2 fixed per O_2 consumed) increased 10- to 20-fold when the partial pressure of oxygen (pO_2) was decreased from 0.21 to 0.01 atm (21.27 to 1.01 kPa). When nitrogenase activity was plotted against pO_2, a typical bell-shaped curve was obtained (61), suggesting that the rate of nitrogen fixation is in fact suboptimal under ambient atmospheric pO_2 values. All this was particularly surprising for *Azotobacter* spp., which were known to depend on oxidative metabolism in support of nitrogen fixation. Lastly, the direct proof of the oxygen sensitivity of nitrogenase came from studies with purified preparations of the enzymes; they were equally destroyed by oxygen, whether they were obtained from an obligate anaerobe, such as *Clostridium pasteurianum* (44), or from the distinctly aerobic *Azotobacter vinelandii* (36, 37).

Autotrophic nitrogen-fixing organisms are no less sensitive to oxygen (106). Most photosynthetic bacteria fix nitrogen only in the light under stringent anaerobic conditions. An exception to this is *Rhodobacter capsulatus* (*Rhodopseudomonas capsulata*), which is able to maintain nitrogenase activity, although at a very low rate, when exposed to air (209, 360). The nonheterocystous cyanobacterium *Plectonema boryanum* requires continuous sparging with an anaerobic gas mixture to prevent inactivation of nitrogenase by the oxygen produced in photosynthesis (321). Nitrogenase activity in all heterocystous cyanobacteria is significantly increased with decreasing concentrations of dissolved oxygen in the culture medium (318). Nitrogenase extracted from cyanobacteria was shown to be highly sensitive to oxygen (92, 150). *Xanthobacter autotrophicus* can fix nitrogen under air both when reducing CO_2 with H_2 as the energy source in a chemoautotrophic metabolism and when grown heterotrophically with sucrose as the carbon and energy source. In the latter case, however, the relationship of nitrogen-fixing activity to pO_2 is similar to that observed with *Azotobacter* species (60).

Studies with symbiotic systems have confirmed the findings made with free-living nitrogen-fixing organisms. ^{15}N fixation by excised soya bean nodules has been found to increase with increasing pO_2 up to 0.5 atm (50.6 kPa) and then decline abruptly at higher oxygen tensions (14, 40). Similarly, ^{15}N incorporation in nonlegume root nodules was shown to be inhibited at higher oxygen concentrations (23, 294). It is thus reasonable to conclude that an anaerobic existence is more compatible with nitrogen fixation than is aerobic metabolism. This conclusion may support the hypothesis that the nitrogenase system emerged during the early period of the Earth's history, when oxygen was most probably absent in the primitive atmosphere (31, 287, 311). Exposure to oxygen irreversibly inactivates both components of the nitrogenase complex (39, 218, 276). Dinitrogenase reductase (Fe-protein) is particularly sensitive to oxygen, as indicated by its rapid destruction in air; its half-life is only 30 s to 2 min. Dinitrogenase (Mo-Fe-protein) is slightly less susceptible, having a half-life in air of between 4.5 and 10 min. The Mo-Fe-cofactor, however, is even more oxygen labile than dinitrogenase reductase (75, 290).

Oxidation of nitrogenase proteins is probably effected by more reactive oxygen species, such as superoxide, hydrogen peroxide, hydroxyl radicals, or singlet oxygen, generated in the course of oxygen reduction (105, 147, 185, 276). They seem to induce a sequence of changes in the structure of proteins and in the redox state of the 4Fe-4S clusters (276), rendering them incapable of accepting or donating electrons (300). Oxygen may also initiate protease activity, leading to the rapid destruction of nitrogenase proteins (274).

Oxygen not only inactivates and destroys nitrogenase, but also represses nitrogenase synthesis. The minimum pO_2 required to repress nitrogenase synthesis in *Azotobacter* spp. is, however, 20 times higher than that which inactivates nitrogenase (263). Both dinitrogenase and dinitrogenase reductase were absent in cells of *K. pneumoniae* (326) and *A. chroococcum* (275) after exposure to oxygen. That their absence could not have resulted from the continuous destruction of newly synthesized enzymes, but was due to repression by oxygen of nitrogenase synthesis, was indicated by the different kinetics of repression induced by ammonia and by oxygen, respectively.

Nitrogenase synthesis in an ammonia-derepressed mutant of *K. pneumoniae* could still be repressed by oxygen (76). In *A. chroococcum*, oxygen repressed nitrogenase synthesis much more rapidly than ammonia did (275). These findings seem to suggest that the two mechanisms of repression, by ammonia and by oxygen, may act independently (373).

Repression of nitrogenase synthesis by oxygen serves to prevent the wasteful production of enzymes doomed to destruction. Products of the structural genes (*nifH*, *nifD*, and *nifK*) are all controlled by oxygen, and the operon *nifAL* appears to be involved in the regulation. The *nifL* gene product acts as a negative control, preventing the expression of other *nif* operons in the presence of oxygen (67, 179).

Studies with *nif* regulatory mutants of *Rhodobacter cap-*

sulatus have shown that the transcriptional controls by combined nitrogen and by oxygen operate separately and at different levels through molecular mechanisms that sense and signal cellular nitrogen status and oxygen tension (183, 184). The first level of control involves the sensing and signaling of the nitrogen status. Genes associated with second-level oxygen control are activated only under nitrogen-deficient conditions. It is known that the cellular O_2 concentration effects the degree of DNA supercoiling, through the action of the enzymes DNA topoisomerase I and DNA topoisomerase II (DNA gyrase). Anaerobic conditions induce high DNA gyrase activity, resulting in a negatively supercoiled, more relaxed conformation of DNA that enables *nif* gene transcription. It is thought that the *nifLA* promoter can sense the state of chromosome supercoiling (182).

PROTECTION OF NITROGENASE FROM OXYGEN

Although the biochemical characteristics and the highly oxygen-sensitive nature of nitrogenase were shown to be essentially the same in all nitrogen-fixing organisms studied, the mechanisms that protect the enzyme system from the damaging effects of oxygen are rather varied. In many diazotrophs more than one mechanism may be present, and in cyanobacteria a whole range of devices seem to operate in an orchestrated fashion to protect nitrogenase from both atmospheric and intracellular sources of oxygen. The protection mechanisms in cyanobacteria are the main subject of this review and are discussed in further detail below. However, a brief survey of the various adaptive mechanisms that operate in other diazotrophs should provide a pertinent basis for comparison. Obligate anaerobes, such as *Clostridium pasteurianum* and *Desulfovibrio desulfuricans*, are apparently devoid of any specific device to protect their nitrogenase, or indeed any other cell constituents, from the deleterious effects of oxygen. Therefore they can live and fix nitrogen only in the complete absence of oxygen and are limited in their natural distribution to oxygen-free environments. Facultative bacteria, for example *Klebsiella pneumoniae*, *Bacillus polymyxa*, and *Rhodospirillum rubrum*, are able to grow on combined nitrogen in both the presence and absence of oxygen but can fix nitrogen only anaerobically. Microaerophilic bacteria, such as *Azospirillum* species, show a preference for subatmospheric levels of oxygen when fixing nitrogen. They are unable to fix nitrogen at high oxygen tensions or under anaerobic conditions. Finally, aerobic bacteria, represented by the *Azotobacter* species, are capable of growth on dinitrogen in air. Certain strains, however, may display oxygen sensitivity during induction of nitrogenase synthesis. Protective mechanisms have been shown to operate in the last three groups of nitrogen-fixing bacteria.

Controlled Oxygen Diffusion

The simplest device that could reduce the flow of oxygen into nitrogen-fixing cells was thought to be increased slime production; this was reported to take place when *Derxia gummosa* was grown on N_2 (155). The suggestion, however, was not substantiated by observations of other slime-producing diazotrophs (276).

Control of oxygen flow is considered to be the main mechanism for the protection of nitrogenase in root nodule bacteroids (16). It is now well established that leghemoglobin provides low-level oxygen transport within the infected cells of legume root nodules. It facilitates a high rate of oxygen flow essential for energy production and concomitantly maintains low dissolved-oxygen concentrations to protect nitrogenase in the bacteroids. The mechanism is reinforced by the presence of membrane boundaries that provide diffusion barriers and restrict the flow of oxygen and also by the action of hydrogenase (195, 292) (see below).

Respiratory Protection

In aerobic nitrogen-fixing organisms, the requirements for nitrogenase activity are generated in oxygen-dependent respiration under conditions that appear to conflict with the oxygen lability of nitrogenase.

Meyerhof and Burk (210) have noted that the efficiency of nitrogen fixation in *Azotobacter* spp. is uniquely conditioned by pO_2. *Azotobacter* spp. and other aerobic diazotrophs are able to respond to increased concentrations of dissolved oxygen by increasing their rate of respiration, thereby maintaining low levels of intracellular oxygen and protecting their nitrogenase from inactivation (61, 158). This adaptation to changes in pO_2 may take place in response to an oxygen-sensing mechanism (252) and probably involves several components of the respiratory system, such as NADH/NADPH dehydrogenases and cytochrome a_2, as well as the main metabolic pathways (171, 276). At higher oxygen tensions the respiratory response becomes nonlinear (259), which may indicate the involvement of additional protective mechanisms (66, 186). Nevertheless, under natural conditions and within a limited range of dissolved-oxygen concentration, respiratory protection may be sufficient to scavenge excess oxygen and to maintain nitrogenase in a virtually oxygen-free cellular environment (158).

Conformational Protection

Under oxygen-stressed conditions (when the oxygen concentration approaches about 20 μM), nitrogenase in *Azotobacter* species is inactivated. The enzyme system will regain full activity upon removal of excess oxygen without new synthesis of nitrogenase proteins (61, 139). It has been assumed that the observed inactivation (or switch-off) of nitrogenase occurs when the capacity for respiratory oxygen scavenging becomes inadequate to protect the nitrogenase and that the reversible nature of this inactivation implies a transient change in the conformation of the enzyme complex (276).

It is well documented that the protected, oxygen-tolerant form of nitrogenase in *Azotobacter* spp. is the result of an association between nitrogenase proteins and a protective 2Fe-2S protein, also called Shetna's protein II (140, 284). The switch-off is apparently triggered by the oxidation of dinitrogenase reductase, mediated by Shetna's protein II, and is followed by the formation of an oxidized, oxygen-stable complex in which the three components are combined in a defined stoichiometric ratio (74, 344). Restoration of nitrogenase activity (switch-on) is initiated by the reduction of the complex, followed by its dissociation (285, 353). The switch-off–switch-on phenomenon has also been observed in *Klebsiella pneumoniae* and *Rhodopseudomonas* species (125). An alternative interpretation of this phenomenon considers the diversion of electrons from nitrogenase to oxygen or other electron acceptors to be the primary event that sets off the reversible inactivation of nitrogenase (125, 144).

Hydrogenase Activity

As mentioned previously, nitrogen fixation in both free-living organisms and symbiotic systems is accompanied by a variable amount of hydrogen evolution in a reaction catalyzed by nitrogenase (276). In the absence of a suitable substrate, such as N_2, nitrogenase discharges protons to evolve H_2 in a reaction that consumes both ATP and reductant (36). H_2 formation appears to be an intrinsic characteristic of the nitrogenase reaction and continues at a low level (1 mol of H_2 per mol of N_2) even under highly elevated oxygen tensions or in the presence of alternative substrates of nitrogenase (acetylene, cyanide, or azide). H_2 evolution, however, is absent in cells grown on ammonia or nitrate, which also suppress nitrogenase synthesis (276). The energy lost in H_2 production is partially regained by the consumption of H_2 in the oxyhydrogen or Knallgas reaction. This is catalyzed by the unidirectional uptake hydrogenase, which appears to be universally present in aerobic nitrogen-fixing organisms. Its activity is strictly oxygen dependent. The rate of oxyhydrogen reaction may approach the rates of endogenous respiration (25). According to Dixon (68), the function of uptake hydrogenase is multifold: it removes H_2 inhibitory to N_2 reduction (216), it acts as an oxygen-scavenging device and augments respiratory protection (348), and it reduces the wastage of energy and reducing power inflicted by H_2 production (263).

Enzymes Protecting against Reactive Forms of Oxygen

The production of reactive oxygen species, such as superoxide radical (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical (HO·), results from univalent reduction of O_2 and invariably accompanies aerobic respiration and oxygenic photosynthesis (105, 153, 185). The formation of these species is directly related to cellular pO_2. Reactive forms of oxygen are extremely toxic to biological systems and would seriously damage not only nitrogenase but also many other essential cell constituents were there not enzymic mechanisms affecting their destruction.

Superoxide dismutase, which catalyzes the reduction of superoxide radicals, is considered to be the primary defense mechanism against potential oxygen toxicity (153). Hydrogen peroxide can be eliminated by the action of catalase, which mediates its conversion to H_2O and O_2. Peroxidases, such as ascorbate peroxidase or glutathione peroxidase, reduce hydrogen peroxide and reinforce the action of catalase. Antioxidant enzymes appear to play an important part in complementing other devices in the protection of nitrogenase against oxygen inactivation in aerobic and microaerophilic diazotrophs (66, 331, 340).

APPARENT INCOMPATIBILITY OF PHOTOSYNTHESIS AND NITROGEN FIXATION IN CYANOBACTERIA

"In completing one discovery we never fail to get an imperfect knowledge of others of which we could have no idea before, so that we cannot solve one doubt without creating several new ones."

Joseph Priestley
Experiments and Observations on Different Kinds of Air (1786)

The role of the various protective mechanisms that have developed in free-living bacteria and in symbiotic nitrogen-fixing systems is to prevent the inactivation and destruction of nitrogenase by oxygen and to enable nitrogen fixation to take place under ambient atmospheric conditions. Essentially these mechanisms protect nitrogenase from the oxygen stress of their natural environment.

Particular Problem of Nitrogen Fixation in Cyanobacteria

The coexistence of oxygen-evolving photosynthesis and oxygen-sensitive nitrogen fixation in diazotrophic cyanobacteria appears to be a remarkable evolutionary achievement, especially when one considers that the two antagonistic processes may occur not simply in the same organism but indeed in the same cell. In the course of evolution, these organisms have had to acquire efficient devices to protect nitrogenase first from oxygen generated as a result of their photosynthetic metabolism and later also from external oxygen stress. The former may be even more imperative when photoevolution of oxygen takes place in the proximity of nitrogenase activity.

It has been established during the past 15 years of intensive research that cyanobacteria have developed a variety of mechanisms to protect their nitrogenase from oxygen (10, 107, 141, 176). These range from relatively simple strategies adapted by certain unicellular cyanobacteria to the most elaborate and efficient mechanisms represented by the specialized nitrogen-fixing cell, the heterocyst. There is plausible indirect evidence that this progression from simple to increasingly more complex devices is consistent with the evolutionary course of the group, probably brought about by a gradual increase in the oxygen content of the atmosphere (31, 123, 159, 286, 287) (see below).

Stages in Detection of N_2-Fixing Potential in Cyanobacteria

The late appreciation of the extreme oxygen sensitivity of nitrogenase and of the varied abilities of diazotrophic cyanobacteria to protect their nitrogenase from inactivation by oxygen is reflected in the slow progress made by investigators for over 50 years in detecting the potential for N_2 fixation in many members of the group.

A suggestion that cyanobacteria might be able to fix N_2 was first put forward by Frank in 1889 (104), but conclusive proof of N_2 fixation in pure cultures of two heterocyst-forming cyanobacteria was first presented 40 years later in 1928 (71). By the mid-1950s, over 20 species, all belonging to heterocystous genera, were shown in critical tests to possess the ability to fix N_2, but no evidence of N_2 fixation in the more than 15 unicellular and filamentous nonheterocystous strains had been obtained during testing under conventional batch culture conditions (102). These findings, and the clear correlation between the presence of heterocysts and the ability to fix N_2, led to the assumption that the potential for N_2 fixation is limited to heterocyst-forming species (98). It was, however, only in 1968 that a definite proposal was made by Fay et al. (94) and shortly thereafter that experimental evidence was presented by Stewart and coworkers (320) suggesting heterocysts as the actual sites of N_2 fixation in these organisms. Differentiation of a vegetative cell into a heterocyst was shown to be essential for the provision of an anaerobic environment and for the protection of nitrogenase from inactivation by both atmospheric and photosynthetic sources of oxygen (188).

In 1961 Dugdale et al. (72) reported on N_2 fixation by planktonic populations of the marine nonheterocystous cyanobacterium *Trichodesmium* (*Oscillatoria*) sp. Although $^{15}N_2$ incorporation had been demonstrated in field material,

TABLE 2. Distribution of nitrogenase activity among nonheterocystous cyanobacteria[a]

Section	Genus	No. of strains tested	No. of positive strains	Fraction of total that were positive
I	*Gloeobacter*	1	0	0
	Gloeothece	5	5	1.00
	Synechococcus	27	3	0.11
	Gloeocapsa	4	0	0
	Synechocystis	16	0	0
II	*Chamaesiphon*	2	0	0
	Dermocarpa	6	2	0.33
	Dermocarpella	1	0	0
	Chroococcidiopsis	8	8	1.00
	Myxosarcina	2	1	0.50
	Xenococcus	3	1	0.33
	Pleurocapsa	12	7	0.58
III	*Spirulina*	2	0	0
	Oscillatoria	9	5	0.55
	Pseudanabaena	8	4	0.50
	Lyngbya-Plectonema-Phormidium group	25	16	0.64
Total		131	52	0.40 (40%)

[a] Reprinted with permission from Rippka and Waterbury (273).

the claim could not be substantiated since the isolation of the strain in axenic culture was unsuccessful. In 1969 Wyatt and Silvey (370) were the first to demonstrate N_2 fixation by a unicellular cyanobacterium of the genus *Gloeocapsa* (later identified as *Gloeothece*) under ambient atmospheric conditions. In 1970 Stewart and Lex (321) showed that the filamentous nonheterocystous *Plectonema boryanum* can synthesize nitrogenase in the light under microaerobic conditions, i.e., when the culture suspension is flushed with a continuous stream of N_2-CO_2 to dispel the oxygen produced during photosynthesis. Almost 10 years later came the report (247) of aerobic N_2 fixation by the filamentous nonheterocystous *Microcoleus chthonoplastes*.

Rippka and Waterbury (273) have developed a method of "anaerobic nitrogenase induction," which allows the expression of nitrogenase in the complete absence of oxygen. It consists of a preliminary treatment of nitrogen starvation (58), which effects the accumulation of reserve carbohydrate, and is followed by continuous flushing of the culture suspension with an anaerobic gas mixture (Ar-CO_2) in the presence of 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) to suppress photosynthetic oxygen evolution. Cyclic photophosphorylation can continue to generate ATP, while the reductant for nitrogenase can be provided by the breakdown of storage glycogen during the experimental treatment. By using this method, they have screened a large number of nonheterocystous cyanobacteria for nitrogenase activity and found that 52 of 131 (40%) synthesized nitrogenase under these conditions (Table 2).

The genetic potential for N_2 fixation is therefore much more widespread among nonheterocystous species than was presumed earlier. Clearly, it is the repression of nitrogenase synthesis by oxygen and the lack or limited ability of the organisms to protect nitrogenase from inactivation by oxygen that prevent the expression of *nif* genes in most nonheterocystous cyanobacteria in aerobic environments.

Facultative Anoxygenic Photosynthesis and N_2 Fixation

Several strains of nonheterocystous cyanobacteria possess the ability to alternate their carbon metabolism between the usual oxygenic photosynthesis and an anoxygenic form of photosynthesis, similar to that operating in photosynthetic bacteria (237). In these strains, sulfide, and not water, is used as the external electron donor and PS I generates both energy and reductant for CO_2 fixation.

The original discovery was made with *Oscillatoria limnetica*, a species isolated from sulfide-rich layers of Solar Lake, Israel (56). In the presence of sulfide concentrations between 0.1 and 0.2 mM, oxygenic PS II-driven photosynthesis is arrested and, after a short (2-h) induction period, anoxygenic PS I-dependent photosynthesis, which is insensitive to the presence of DCMU, begins. In the absence of CO_2, PS I-driven sulfide oxidation results in hydrogenase-catalyzed hydrogen evolution. When sulfide is removed, the organism immediately reverts to oxygenic photosynthesis, which seems to indicate that PS II, although inhibited by sulfide, remains constitutively present in the cells of *O. limnetica* (235).

Screening 21 strains of cyanobacteria for the ability of facultative anoxygenic photosynthesis, Garlick et al. (118) found 1 unicellular and 10 filamentous nonheterocystous strains that were able to shift their photochemical mechanism to the PS I-driven sulfide-dependent type of CO_2 fixation. When anoxygenic photosynthesis is induced in the absence of combined nitrogen, *O. limnetica* is also able to synthesize nitrogenase and to reduce acetylene (11). As for CO_2 fixation, sulfide functions as an electron donor, while the ATP required for N_2 fixation is generated by PS I.

Although the conditions of sulfide-dependent anoxygenic photosynthetic metabolism may appear most favorable for nitrogenase activity, it is rather surprising that no other case of a similar association between anoxygenic photosynthesis and nitrogen fixation in cyanobacteria has so far been detected and reported. The reasons for this are unknown.

PROTECTION OF NITROGENASE FROM OXYGEN IN NONHETEROCYSTOUS CYANOBACTERIA

Research in the past two decades has failed to discover in nonheterocystous cyanobacteria the existence of a universal system for the protection of nitrogenase against inactivation by oxygen. Instead, there appear to be a variety of strategies that function more or less efficiently, alone or in combination, to protect the enzyme complex against both exogenous (atmospheric) and endogenous (photosynthetic) sources of oxygen. A range of oxygen tolerances has emerged from these studies. It is noteworthy that all the strains that have been investigated in some detail have displayed a particular response to oxygen under nitrogen-fixing conditions. A brief discussion of these responses, as revealed with a few representative strains of nonheterocystous nitrogen-fixing cyanobacteria, is necessary before an attempt is made to draw some general conclusions.

Gloeothece

Since Wyatt and Silvey (370) first reported that a *Gloeothece* sp. is capable of aerobic N_2 fixation, many attempts have been made to find an explanation for how nitrogenase activity is maintained under air and apparently in the same cells that simultaneously liberate O_2 in photosynthesis. This was particularly puzzling because it was shown that nitro-

genase in *Gloeothece* sp. is readily inactivated at higher O_2 tensions (111, 208) and that growth is particularly slow (generation time, between 40 and 100 h) at higher irradiances (above 500 to 1000 lx), which enhance photosynthetic O_2 evolution (112, 113, 271). Light- and electron-microscopic examination failed to detect any morphological or structural differences between cells grown on dinitrogen and those grown on combined nitrogen (271). However, the existence of protective mechanisms was strongly indicated by the observation that nitrogenase in *Gloeothece* sp. was much more sensitive to O_2 when it was induced anaerobically rather than in air, suggesting that the expression of protective mechanisms requires the presence of O_2 (176).

Gloeothece cells are surrounded by an elaborate sheath, and it was suspected that this might constitute a passive barrier to the diffusion of O_2, thereby protecting nitrogenase against inactivation by O_2. Comparison of the effects of O_2 on nitrogenase activity of the wild-type sheathed, artificially unsheathed, and sheathless mutant strains showed, however, no significant differences and thus failed to support the idea that the sheath has a role in the protection of nitrogenase from oxygen (176).

The first proposals regarding possible temporal separation of photosynthetic and nitrogen-fixing activities were based on the finding that these two activities peaked at different stages during growth in continuously illuminated batch cultures (112, 113). Such variations in the rates of nitrogen assimilation and protein synthesis, and of carbon metabolism and carbohydrate accumulation, are, however, also common in batch cultures of various non-N_2-fixing photosynthetic microorganisms (101). More revealing are studies by Gallon and colleagues on the variations in metabolic activity in cultures grown with alternating light-dark periods under conditions that resemble the natural diurnal cycle (108, 221). When *Gloeothece* cells were incubated under a 12-h light–12-h dark regime, significant nitrogenase activity was detected only during the dark period, and it was apparently supported by the catabolic breakdown of reserve carbohydrate accumulated in the previous light period (49, 109, 114, 220) (Fig. 1). This latter interpretation is compatible with the observation that rates of nitrogenase activity in the dark are directly related to the quantity of irradiance received by the cells in the light. Also, nitrogenase activity was inhibited by potassium cyanide but not by DCMU and was evidently O_2 dependent, with an optimum dissolved-O_2 concentration of about 52 μM (77). The observations led to the conclusion that aerobic respiration and not photosynthesis is the main source of reductant and energy for N_2 fixation in *Gloeothece* sp. both in the dark and in the light (77, 207). In studies with well-aerated *Gloeothece* continuous cultures, Ortega-Calvo and Stal (236) have observed that nitrogenase activity is light stimulated and occurs mainly during the light period. They have concluded that temporal separation of N_2 fixation and photosynthesis is not obligatory for diazotrophic growth of the organism and that the two activities can occur simultaneously.

The possible role and significance of additional mechanisms that may reinforce the protection of nitrogenase in *Gloeothece* from inactivation by O_2 remain obscure. Since nitrogenase activity is dependent on respiration, it should no doubt be enhanced by the concurrent decrease in cellular O_2 tension (111, 220). Reports on the presence of uptake hydrogenase (342), but not of catalase (340), and that a nonenzymatic reaction with ascorbate and an enzyme-catalyzed reaction with glutathione affect the removal of hydrogen peroxide (340) are noteworthy although short of quantitative

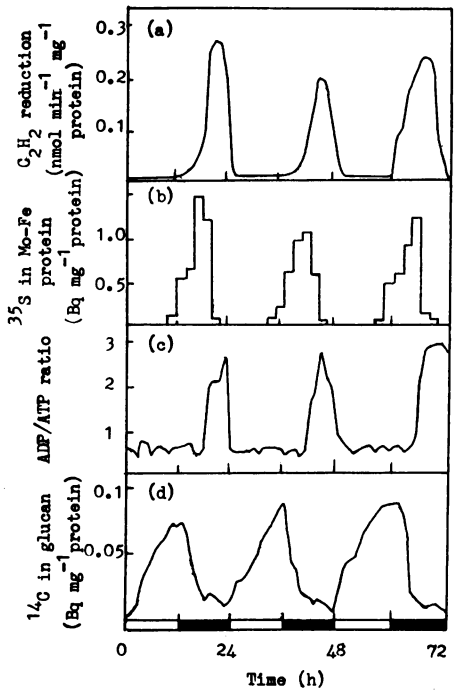

FIG. 1. Nitrogenase activity (a), nitrogenase synthesis (b), ADP/ATP ratio (c), and radioactivity (content) of intracellular glucan (d) in *Gloeothece* sp. strain ATCC 27152 incubated under an alternating cycle of 12 h of light (25 μmol of photons m^{-2} s^{-1}) and 12 h of darkness. Adapted with permission from Gallon et al. (114).

evaluation of their expected function. Transfer of *Gloeothece* cells from air to O_2 causes a transitory repression of nitrogenase synthesis, which is resumed upon prolonged incubation under O_2 (208). Studies on DNA topoisomerase activities suggest that the transient repression of nitrogenase synthesis may result from a transient relaxation of *nif* genes (109, 110).

Synechococcus

Nitrogen fixation in one freshwater and two marine strains of the unicellular genus *Synechococcus* has been investigated in different laboratories.

The fast-growing (generation time, 20 h) marine *Synechococcus* strains, Miami BG43511 and Miami BG43522, have been shown to synthesize nitrogenase under conditions of nitrogen depletion and to maintain enzyme activity during aerobic incubation (214). When synchronous growth was induced (by transfer in the dark and aeration for 20 h) and then the synchronized cell material was incubated under alternating 12-h light–12-h dark cycles, metabolic activity and cell growth and division displayed regular cyclic variations. Photosynthesis peaked in the light, whereas maximum nitrogenase activity was measured in the middle of the dark period. The cell carbohydrate content was highest in the middle of the light phase, its rapid decrease in the dark coinciding with increased nitrogenase activity. This was thought to imply that nitrogen fixation depended on the degradation of cellular carbohydrate reserves and that energy, reductant, and possibly protection of nitrogenase from oxygen were provided by means of respiration. Cell division regularly occurred at the beginning of the light period. When the synchronized cell material was subsequently incubated

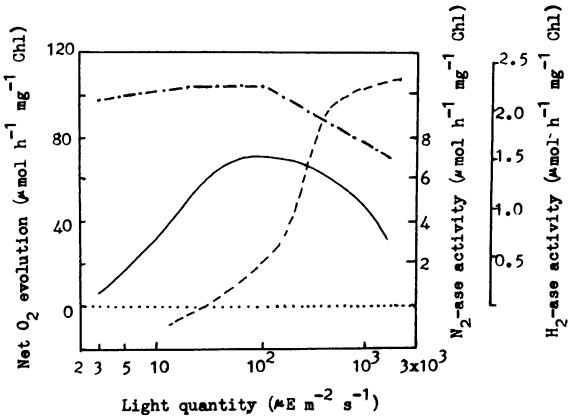

FIG. 2. Effect of light quantity on O_2 evolution (broken line), nitrogenase activity (solid line), and hydrogenase activity (dash-dotted line) of *Synechococcus* sp. strain SF1. Adapted with permission from Spiller and Shanmugam (310).

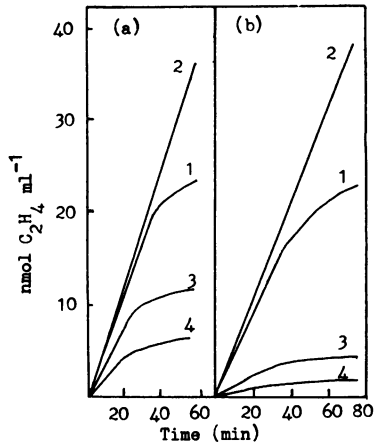

FIG. 3. Effect of O_2 on nitrogenase activity of *P. boryanum*. (a) Samples of a culture grown under 99.7% N_2–0.3% CO_2 were incubated in light (6×10^{-4} erg cm^{-2} s^{-1}) under Ar (curve 1), Ar-DCMU (curve 2), 0.8% O_2 (curve 3), and 1.7% O_2 (curve 4), respectively. Nitrogenase activity was assayed at intervals to determine O_2 inhibition. (b) Suspension samples were then flushed with N_2-CO_2, acetylene was added, and gas samples were withdrawn at intervals for acetylene reduction assay to determine O_2 inactivation of nitrogenase. Adapted with permission from Weare and Benemann (356).

under continuous illumination, the synchrony was apparently sustained for three further cell division cycles (196). In the course of these cycles, little or no nitrogenase activity was detected during periods of cell division, active photosynthesis, cell elongation, or carbohydrate accumulation. The decline in photosynthetic O_2 evolution coincided with the increase in nitrogenase activity and the depletion of reserve carbohydrate. Mitsui et al. (213) concluded that temporal segregation of photosynthesis and nitrogen fixation within the cell division cycle enables *Synechococcus* strains to grow on dinitrogen in the natural diurnal cycle.

Another, rather slow-growing (generation time, about 60 h) epiphytic marine isolate of the genus *Synechococcus*, designated strain SF1, seems to differ in several aspects from the Miami strains, most importantly in its inability to fix N_2 in the dark (310). Its nitrogenase showed a distinct sensitivity to O_2, with an optimum O_2 concentration of about 1% in the gas phase (0.01 atm [1.01 kPa]). Induction of nitrogenase synthesis required anaerobic treatment of the culture and was promoted by a high density of the cell suspension. Essentially, the condition corresponded to low photon fluence density and thus to decreased rates of photosynthetic O_2 evolution. The optimum light flux for N_2 fixation (40 microeinsteins m^{-2} s^{-1}) was near the compensation point for photosynthesis. Uptake hydrogenase activity was coinduced with nitrogenase activity. Its possible role in protecting nitrogenase from inactivation by O_2 is indicated by the correlation between the rates of the two enzyme activities (Fig. 2).

The freshwater *Synechococcus* species strain RF1, isolated from a rice field, grows slowly in culture (generation time, about 60 h) and can fix N_2 aerobically, both when illuminated continuously and when incubated in alternating light-dark cycles (12 h light, 12 h dark) (166). Nitrogenase activity in the latter case was always restricted to the dark period. Rates of acetylene reduction were relatively low when cultures were grown under continuous illumination and were increased almost threefold when dissolved-O_2 concentrations were reduced by sparging cultures with N_2 or by the addition of DCMU (137). The rhythmic occurrence of nitrogenase activity observed with cultures adapted to a diurnal regime was maintained when cultures were changed to continuous illumination (136). Nitrogenase activity was inversely related to dissolved-O_2 concentration, and maximum nitrogenase activity coincided with a distinct fall in net O_2 evolution resulting from increased rates of respiration. During growth in a diurnal cycle, the synthesis both of nitrogenase and of nitrogenase mRNA occurred in the dark (167, 168). This cyclic pattern of *nif* gene expression was sustained for several days when the culture was changed to continuous illumination (167).

Ascorbate peroxidase, the enzyme involved in the removal of H_2O_2, has been shown to be present in *Synechococcus* sp. strain PCC7942, which is unable to fix N_2 (280). Enzyme activity and its potential protective function depend on the presence of ascorbate and are increased with increasing concentrations of H_2O_2.

Plectonema

The filamentous nonheterocystous *Plectonema boryanum* is able to fix N_2 when incubated in the light under microaerobic conditions, as effected by sparging the culture suspension continuously with an O_2-free gas mixture (N_2-CO_2) (246, 278, 321, 356). The time required for enzyme induction (i.e., between the transfer of filaments to nitrogen-free medium and the onset of nitrogenase activity) depended on factors that directly or indirectly affect the levels of exogenous and endogenous O_2. Thus the lag was increased with increasing irradiation (119) and diminished in the presence of DCMU (356). Sustained concomitant photoautotrophic growth and N_2 fixation has been achieved by conditions such as exclusion of O_2 (246), low photon fluence rate (119), or the application of chemical reductants and O_2 absorbers such as sulfide and dithionite (246, 278). All these observations provide evidence for the exceptionally high O_2 sensitivity of nitrogenase in *Plectonema* sp. or, more exactly, the lack of any apparent mechanism to protect the enzyme system from inactivation by O_2 (Fig. 3).

P. boryanum is able to grow heterotrophically in the dark under air in the presence of suitable organic substrates such as glucose (358). When sulfide was supplied at a concentra-

tion that inhibited CO_2 fixation (278) or when CO_2 was excluded from the culture (306), photoheterotrophic growth on N_2 could be sustained at the expense of organic carbon sources in the medium. Under these conditions, reductant for N_2 fixation is apparently generated by oxidative metabolism while cyclic photophosphorylation may provide the ATP requirement for nitrogenase activity (246). Limited N_2 fixation can also take place in the dark (356) by utilizing endogenous carbon reserves (accumulated during the light period) at low pO_2 (0.5% of the gas phase, or less), sufficient to support respiratory metabolism but not to inactivate nitrogenase. Aerobic respiration could not only meet the requirements for reductant and energy, but could also provide limited protection for nitrogenase by the control of intracellular O_2 tensions. A proposal regarding temporal separation of photosynthesis and N_2 fixation, based on the observation of alternating peaks of photosynthetic and nitrogenase activities and on a rhythmic cycle of degradation and synthesis of phycobiliprotein pigments (356), was not substantiated in subsequent studies (119, 246). Using an immunogold localization technique, Smoker and Barnum (307) demonstrated the presence of nitrogenase in all cells of the *Plectonema* trichome, but only under N_2-fixing conditions. In contrast, the key photosynthetic enzyme, Rubisco, was present in both N_2-fixing and nonfixing filaments, although at a reduced quantity in cells that also contained nitrogenase.

Microcoleus

The filamentous *Microcoleus chthonoplastes*, isolated from a marine cyanobacterial mat, has been shown to reduce acetylene under aerobic conditions (247). The species displays a tendency for aggregation, i.e., the formation of bundles or tightly coiled ropes. Light- and electron-microscopic examination could not reveal any signs of cell differentiation under N_2-fixing conditions. Reducing potential, when tested by the triphenyltetrazolium chloride reaction, was found to be similar in all cells along the trichome (204).

Although *M. chthonoplastes* was able to fix N_2 under ambient atmospheric conditions, reduced O_2 tensions increased its nitrogenase activity in the light. However, in the dark, complete deprivation of O_2 prevented acetylene reduction (247). This and the dependence of nitrogenase activity in the dark on carbon reserves produced by photosynthesis during previous light incubation (248) imply that the energy and reductant required for N_2 fixation are generated in aerobic respiration. Since the highest rates of nitrogenase activity were measured in the light (248), it is assumed that *M. chthonoplastes* must possess adequate devices to protect their nitrogenase against inactivation by O_2 released in photosynthesis. The nature of these devices is as yet unknown.

It has been reported that, in the presence of DCMU, *M. chthonoplastes* can perform anoxygenic photosynthesis associated with the oxidation of sulfide to thiosulfate (64). It is also capable of fermentative metabolism upon transfer to dark anaerobic conditions (215). There is no report on N_2 fixation under such conditions.

Oscillatoria

Investigations of N_2 fixation by the marine *Oscillatoria limosa* strain 23, undertaken by Stal and Krumbein and their associates, have revealed several features that could be important in the survival of cyanobacteria in the specific benthic environment of intertidal mats, from where this strain was isolated (314). Close examination of the morphology and pigmentation of the trichomes showed no indication of cell differentiation. In a recent study by Stal and Bergman (312), in which immunogold electron microscopy was used to localize the Fe-protein of nitrogenase, it was shown that the protein is distributed evenly throughout the cytoplasm and that it is present in all cells of the trichome under N_2-fixing conditions. The ability of the organism to maintain nitrogenase activity under air must therefore be attributed to the existence of certain mechanisms that may protect the enzyme against inactivation by O_2. Nitrogenase activity was shown to be unaffected by O_2 concentrations up to 0.15 atm (15.19 kPa), but higher O_2 tensions were inhibitory and eventually caused the inactivation of nitrogenase (316), apparently because of the limited capacity of the O_2-scavenging devices. Distinct sensitivity of nitrogenase to O_2 was indicated by the response to decreased atmospheric and endogenous O_2 tensions. Thus nitrogenase activity was enhanced 4.5-fold when filaments were incubated under helium, 6-fold when DCMU was added to the culture suspension, and 5-fold when cultures were transferred in the dark (314). A combination of low photon fluence rate, the presence of DCMU, and anaerobic incubation provided the highest rates of acetylene reduction (Table 3).

TABLE 3. Effect of O_2 and light on nitrogenase activity of *Oscillatoria* sp. strain 23[a]

Conditions of incubation	Addition	Acetylene reduction (nmol of C_2H_4 produced/mg of protein/h)
Aerobic,[b] light[c]		92
Anaerobic,[d] light		389
Aerobic, light	DCMU[e]	488
Anaerobic, light	DCMU	1,332
Aerobic, dark		415
Anaerobic, dark		58

[a] Reprinted with permission from Stal and Krumbein (314).
[b] 20% O_2 in He.
[c] 4 W m^{-2}.
[d] 100% He.
[e] 10^{-5} M.

When *O. limosa* was grown with alternating light-dark cycles, nitrogenase activity was measurable mainly during the dark period (317). This was thought to indicate the temporal separation of photosynthetic and N_2-fixing activities under such conditions (315). Changing from light to dark incubation resulted in the cessation of O_2 evolution and increased rates of respiration (317). Reductant and ATP required for N_2 fixation were apparently generated at the expense of catabolic breakdown of reserve carbohydrate accumulated during the light period. High respiratory activity concomitant with decreased pO_2 would elevate N_2 fixation.

Nitrogenase activity was not detectable when chloramphenicol was added to the culture 3 h before the beginning of the dark period. This was assumed to suggest that nitrogenase may be newly synthesized shortly before the end of the light period, when *O. limosa* was grown under an alternating light-dark regime (315). On the other hand, inactivation of nitrogenase caused by the sudden exposure of cultures to high pO_2 was found to be relieved when the pO_2 was lowered, even in the presence of chloramphenicol. This observation has been interpreted to imply that no new synthesis of the enzyme occurred, but that some kind of switch-off mechanism or conformational protection of nitro-

genase might be involved (316). The two seemingly contrasting observations on the effect of O_2 on nitrogenase of *O. limosa*, as well as the finding that elimination of photosynthetic O_2 production (by DCMU or upon transfer in the dark) has allowed a greatly increased rate of enzyme activity, appear to indicate that photoevolution of O_2 may be more detrimental to nitrogenase located in the same cell than a rise in external O_2 tension. This conclusion is compatible with the proposal of increased nitrogenase synthesis during light incubation, which would serve to compensate for possible enzyme losses caused by photosynthetically elevated intracellular O_2 concentrations (316). Further research is needed to test this hypothesis.

Significant nitrogenase activity was also recorded when *O. limosa* was incubated under dark anaerobic conditions. This was considered to indicate that requirements for N_2 fixation can be met in this organism through fermentative metabolism (313). Indeed, Heyer et al. (154) demonstrated that *O. limosa* is able to degrade glycogen, glucose, and trehalose along fermentative pathways when incubated in the absence of O_2 in the dark. The energy thereby generated might be sufficient to meet maintenance requirements but is probably inadequate to support N_2 fixation and growth.

Field measurements of photosynthetic and nitrogenase activities in marine cyanobacterial mats, dominated by *Oscillatoria* or *Microcoleus* spp., have indicated that temporal separation of the two metabolic processes also occurs in natural populations of cyanobacteria (9, 65, 346). The diurnal patterns, however, are influenced by seasonal variations in light intensity, temperature, and dissolved-O_2 concentration (346).

Trichodesmium

Strains of gas-vacuolated marine planktonic *Oscillatoria* (*Trichodesmium*) are widely distributed in the tropical and subtropical oceans, periodically forming massive blooms under calm water conditions. *Trichodesmium* species are considered to be the most important agents of N_2 fixation in the marine pelagic environment (99). Populations were found to peak at a depth of 15 to 25 m below the surface, and a significant proportion of the population may occur in deeper waters, down to 100 to 200 m (47). *Trichodesmium* cells are certainly adapted to the hydrostatic pressures in their natural environment, as evidenced by the extreme strength of their gas vesicles (349, 352). The buoyancy provided by the gas vesicles enables the organisms to occupy a favorable position in the euphotic zone; it also allows a diel vertical migration of filaments, which is regulated by a carbohydrate ballasting mechanism (345). The buoyancy regulatory mechanism may become disturbed under calm water conditions, causing surface accumulation of large populations and the formation of surface blooms. The exposure of cells to highly unfavorable conditions at or near the water surface results in cell lysis and mass destruction.

Research on the physiology of *Trichodesmium* spp. and on their remarkable ability to maintain nitrogenase activity in seawaters saturated with O_2 has been hampered until recently (see below) by the repeated failure to isolate and grow the organism in axenic culture. Our knowledge is therefore based mostly on studies undertaken with freshly collected planktonic material. Evidence of the ability of *Trichodesmium* spp. to fix N_2 is borne out by the observed light stimulation of nitrogenase activity (34, 281), by the significant incorporation of $^{15}N_2$ in the *Trichodesmium* fraction rather than in any other accompanying particulate fractions (34), by immunocytochemical localization of nitrogenase in *Trichodesmium* cells (17, 244), and by the similarity of the *nifH* segment of DNA (isolated from a natural assemblage of *Trichodesmium* spp.) to the *Anabaena nifH* gene (375).

In the field material, active N_2 fixation appeared to be associated with aggregation of trichomes in parallel planes into characteristic bundles. Disruption of bundle morphology resulted in the partial or total loss of nitrogenase activity (34, 46, 282). Using ^{14}C radioautography, Carpenter and Price (46) have observed that certain lightly pigmented cells situated in the center of the colony lack CO_2-fixing activity. By assuming that O_2 evolution is also absent in the same cells, it has been proposed that the cells might be specialized for N_2 fixation in a similar way to heterocysts in heterocystous cyanobacteria. It was thought that the O_2 concentration in these cells may be low enough to allow nitrogenase to function. The hypothesis was supported by the observation that cells in the central region of the trichomes display high reducing potential (34, 242). The regular presence of rod-shaped heterotrophic bacteria in close association with trichomes in the central core of bundles (34, 241) could further promote the maintenance of microaerobic conditions. Measurements with an oxygen microelectrode have established a good correlation between O_2-depleted microzones inside the bundles and nitrogenase activity (240, 241). H_2 production by *Trichodesmium* spp. has been reported (282), but there seems to be no information on the presence and possible role of an uptake hydrogenase. Results from field studies on diurnal variations of nitrogenase activity of *Trichodesmium* spp. are consistent with a distinct ability for aerobic N_2 fixation, but also with an apparent sensitivity of its nitrogenase to elevated intracellular O_2 tensions (34). Under clear-sky conditions, the enzyme activity increased rapidly during the early morning period, reaching a maximum several hours prior to the midday peaks of solar radiation and photosynthetic O_2 evolution. Light evidently stimulated nitrogenase activity, possibly through an ample supply of ATP, but higher irradiances suppressed enzyme activity, probably by increased photoproduction of O_2. Nitrogenase activity was rather low but remained measurable throughout the nocturnal period in this study but not in a more recent investigation by Capone et al. (41). These authors have suggested that activity is "turned off" in the evening and "turned on" in the early morning.

Ohki and Fujita (231) have succeeded in isolating and maintaining *Trichodesmium* spp. in laboratory culture by eliminating grazers and other associated microorganisms and by taking account of the exceptional sensitivity of the organism to heavy metals and of its requirement for relatively high concentrations of Ca^{2+}. Clonal cultures were raised from single trichomes. Although no other N_2-fixing bacteria were detected in these cultures, their axenic nature is questionable (Ohki, personal communication). Trichomes remain isolated during the early, log phase of growth, and small bundle-shaped colonies are formed during the late exponential or postexponential phase of growth in culture. Colony formation is apparently not a precondition for N_2 fixation in culture. On the contrary, nitrogenase activity was shown to be inversely related to colony size (232). Activity, which is little affected by O_2 concentrations below 0.1 atm (10.13 kPa), is light dependent and suppressed by DCMU; it is highest during the log phase of growth and insignificant in the dark (232, 233). When *Trichodesmium* spp. were grown under a light-dark regime, nitrogenase activity was significant only in the light, almost disappeared in the dark, and

reappeared upon exposure to light. These observations are in agreement with earlier records made with material collected in the field (34, 281).

The concept of segregation of photosynthetic and nitrogen-fixing activities along the trichomes and within the bundles of *Trichodesmium* spp. has been challenged by the results of immunolocalization studies (17, 244). By using an immunogold staining technique to localize nitrogenase in *Trichodesmium* spp., labeling was shown to occur in all cells of the trichome (244) or to be confined to about 10 to 40% of trichomes within the bundle (17). No qualitative differences could be detected by means of epifluorescence microscopy in the pigment composition of cells along the trichomes positioned either in the peripheral or the central region of bundles (45). Neither could a consistently low O_2 tension in the center of colonies be confirmed.

Although nitrogenase activity can be detected only when *Trichodesmium* spp. are grown in a medium free from combined nitrogen, the component proteins of the enzyme complex were shown to be present, in an apparently inactive form, in cells grown on nitrate or in the presence of low concentrations of ammonium, but not urea (234). Western immunoblot analysis of *Trichodesmium* cell extracts indicates that both component proteins are synthesized in cells grown on N_2 and also in cells grown with nitrate or ammonium. Two modifications of the Fe-protein, with apparent molecular masses of 38 and 40 kDa, were detected in cells fixing N_2, but only one, the higher-molecular-mass form, was present in cells raised with nitrate or ammonium; none was detectable in cells grown with urea. The lower-molecular-mass form of Fe-protein was also absent when trichomes were exposed to high concentrations of O_2 and when cultures were incubated in the dark. The observations seem to suggest that the 38-kDa form of Fe-protein corresponds to the active conformation of the enzyme while the 40-kDa form may be the inactive posttranslational modification of the protein (234). It is possible that nitrogenase activity in *Trichodesmium* spp., as in certain heterocystous cyanobacteria (see below), is regulated, and protected, by the reversible modification of the Fe-protein, since this modification arises upon exposure to O_2. When trichomes are incubated under N_2, the enzyme is fully unmodified and presumably most active (374). Since both forms of the Fe-protein seem to be present in trichomes fixing N_2, and indeed in trichomes grown with combined nitrogen, the possibility that the two modifications were derived from two different cells of the trichome (i.e., one actively fixing N_2, the other fixing CO_2 and evolving O_2) cannot be ruled out. The organization of *Trichodesmium nif* genes has been shown to be contiguous, as in other nonheterocystous N_2-fixing cyanobacteria (376). Although the original puzzle of how *Trichodesmium* spp. are able to accommodate the contrasting metabolic activities in the same cells remains unresolved, recent research has opened up new perspectives. Axenic cultures should enable basic physiological and biochemical investigations on the possible presence of mechanisms capable of protecting nitrogenase. Light stimulation of nitrogenase activity in *Trichodesmium* spp. could stem from the direct supply of ATP and reductant generated in the photochemical reaction. Alternatively, it could be the result of light-dependent O_2 consumption or photorespiration, which may play an important part in decreasing cellular O_2 tension and thus in protecting nitrogenase against inactivation by O_2.

General Comments

Studies with nonheterocystous cyanobacteria have revealed no universal strategy, comparable to that represented by the heterocyst, that would allow nitrogenase to function in these organisms under ambient atmospheric conditions and concomitant with oxygenic photosynthesis. The relationship between nitrogenase activity and pO_2, as illustrated by a bell-shaped curve, provides a convenient measure of O_2 sensitivity for an organism under conditions of N_2 fixation. A range of O_2 tolerance is evident within the group. At one end we find organisms such as *P. boryanum*, which seem to lack any particular device to protect nitrogenase and can therefore express their N_2-fixing potential only in the absence of O_2 or in a strongly reducing environment. At the other end are *Trichodesmium* strains, which have apparently acquired some kind of structural and/or biochemical mechanisms that enable N_2 fixation to proceed in waters saturated with O_2, in the light, and concurrent with O_2-evolving photosynthesis. Organisms such as *Gloeothece*, *Synechococcus*, *Microcoleus*, and *Oscillatoria* species seem to occupy intermediate positions of O_2 sensitivity and appear to share certain characteristics that contribute in various degrees to their ability to maintain a functional nitrogenase.

Apart from the controversial situation in *Trichodesmium* spp., it is most likely that nitrogenase is synthesized, under nitrogen-depleted conditions, in all cells of the colony or trichome of nonheterocystous cyanobacteria. This view is consistent with the demonstration that nitrogenase activity in these organisms is particularly susceptible to O_2 generated by photosynthesis within the same cell. Protective devices such as respiratory O_2 consumption could become inadequate in the light, especially at high photon fluence rates, to compensate for photosynthetic O_2 evolution. Nitrogenase is liable to inactivation, even degradation, in strong natural light and may regularly be newly synthesized in dim light or during the nocturnal period. Aerobic respiration, and not photosynthesis, is the main mechanism of generating energy and reductant necessary for N_2 fixation. Aerobic respiration also contributes to the protection of nitrogenase by lowering O_2 tension. Respiration is, however, dependent on reserve carbohydrate accumulated during photosynthesis in an earlier light period. ATP generated by cyclic photophosphorylation may also be available for N_2 fixation in certain strains; in others, fermentation may provide the requirements of limited nitrogenase activity under dark anaerobic conditions.

Little is known of the existence and importance of conformational protection or of other devices that may contribute to the protection of nitrogenase against inactivation by O_2 under aerobic conditions in nonheterocystous cyanobacteria. Temporal separation of photosynthetic and nitrogenase activities appears to be, in most cases, the manifestation of favorable (stimulatory) or hostile (inhibitory) environmental conditions for the contrasting metabolic activities. In certain species, however, the segregation of activities may be regulated by an endogenous rhythmic mechanism, although certain reservations must be made until more direct evidence is available to support the existence and characteristics of such mechanisms.

N_2 fixation by nonheterocystous cyanobacteria in nature is influenced by complex and continuously changing environmental conditions, including the rapid changes in the concentration of dissolved O_2. In the marine microbial mat community, often dominated by nonheterocystous cyanobacteria, O_2-depleted microzones may significantly enhance N_2 fixation (239).

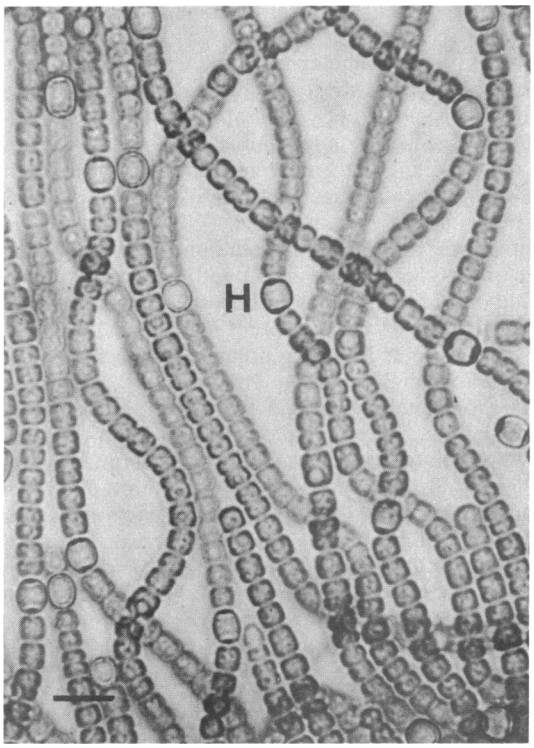

FIG. 4. Light micrograph of *A. variabilis* showing heterocystous (H) filaments. Bar, 10 μm.

PROTECTION OF NITROGENASE FROM OXYGEN IN HETEROCYSTOUS CYANOBACTERIA

Heterocysts are complex cellular systems produced by the transformation of vegetative cells in cyanobacteria belonging to group sections IV and V (270) and comprising the genera *Anabaena*, *Nodularia*, *Cylindrospermum*, *Nostoc*, *Scytonema*, *Calothrix*, *Fischerella*, and *Chlorogloeopsis* (Fig. 4). Heterocysts provide a finely regulated anaerobic microenvironment for the efficient function and protection of nitrogenase. Heterocyst development results in the distinct spatial separation of the two contrasting metabolic activities of oxygenic photosynthesis and oxygen-sensitive nitrogen fixation.

Comprehensive information on the differentiation, morphology, and pattern formation, and on the cellular organization and function of heterocysts is available from several monographs and review articles (2, 89, 100, 145, 364). Although this review concentrates on the discussion of various mechanisms that serve to protect nitrogenase in the heterocysts against damage from oxygen, a brief summary of our present knowledge of the heterocyst may nevertheless be appropriate for readers less familiar with the biology of cyanobacteria.

Heterocyst differentiation involves profound structural and biochemical changes, which include the mobilization of granular inclusions and reserve products, the deposition of a multilayered envelope external to the cell wall, the formation of a narrow junction between the heterocyst and the adjacent vegetative cell, the disintegration and new formation of the intracytoplasmic membrane system, and protein degradation and synthesis of new proteins (96, 97, 188, 194). During a period of nitrogen deficiency, the condition that initiates heterocyst differentiation and nitrogenase synthesis, the cellular nitrogenous reserves are rapidly depleted, raising the cellular carbon/nitrogen ratio from the usual value of about 4.5 up to 8.1. Heterocyst development apparently begins when the ratio rises above 6.1. Trichomes with so-called proheterocysts that are not fully matured show no nitrogenase activity. Activity is detectable only after the carbon/nitrogen ratio increases to about 8.1. This coincides with the appearance of the first mature heterocysts (188). The apparent prerequisite for the expression of nitrogen-fixing potential is the completion of structural and biochemical changes that enable nitrogenase to function effectively and without obstruction by oxygen in the transformed cell. In studies with mutants of *Anabaena* sp. strain PCC7120, Buikema and Haselkorn (35) have recently identified the gene *hetR*, one of several genes that control heterocyst development. The presence of extra copies of this gene leads to the formation of multiple heterocysts. The action and role of the *hetR* gene product in heterocyst differentiation have not yet been established, although it is possible that it is able to sense cellular C/N ratios.

The regular spacing of heterocysts within the trichome can be seen as a rational device promoting the even and efficient distribution of fixed nitrogen from the heterocysts to vegetative cells in a linear multicellular system. Carbon compounds transported in the opposite direction (363) provide substrates for respiration and carbon skeletons to incorporate the nitrogen fixed in the heterocyst (Fig. 5). Present ideas on the pattern of heterocyst differentiation are elaborations on the early hypothesis by Fogg (98) that fixed nitrogen inhibits heterocyst formation and that the source of the inhibitory substance is the heterocyst. The identity of the inhibitor, which is thought to diffuse along the filament, is uncertain (365).

Effect of O_2 on N_2 Fixation in Heterocystous Cyanobacteria

Heterocystous cyanobacteria grow in a variety of habitats where O_2 tensions may fluctuate considerably and could influence their ability to fix N_2 and the rate of N_2 fixation. In common with nitrogenases produced in other bacteria, the nitrogenases of heterocystous cyanobacteria are irreversibly inactivated by O_2 (92, 150, 272).

Anaerobic conditions, applied during the preparation and incubation of cell extracts from cyanobacteria, have greatly increased their N_2-fixing activity. $^{15}N_2$ uptake by cell-free preparations of *Anabaena cylindrica* was lower in the light than in the dark, owing to photoinhibition of nitrogenase activity by photochemically active particles in such preparations (92) (Table 4). The addition of sulfydryl agents increased N_2 uptake by maintaining reducing conditions. Since O_2 rapidly inactivates nitrogenase in isolated heterocysts (95, 253, 320), stringent anaerobic conditions must be applied during their isolation (90).

When cultures of *A. flos-aquae* and *Nostoc muscorum* were incubated in the light under a range of O_2 tensions, the highest rates of nitrogenase activity were measured at subatmospheric pO_2 levels (near 0.1 atm [10.13 kPa]) (323) (Fig. 6). Activity was lower both when O_2 was absent in the gas phase (by about 18%) and under ambient O_2 tensions (by about 38%). Higher than atmospheric O_2 concentrations caused marked inhibition of nitrogenase activity. When the pO_2 was readjusted to atmospheric level, nitrogenase activity recovered at a similar rate for all treatments. High levels of pO_2 could be inhibitory, not only to N_2 fixation but also to respiration and photosynthesis (323).

The response of in vivo nitrogenase activity to O_2 is

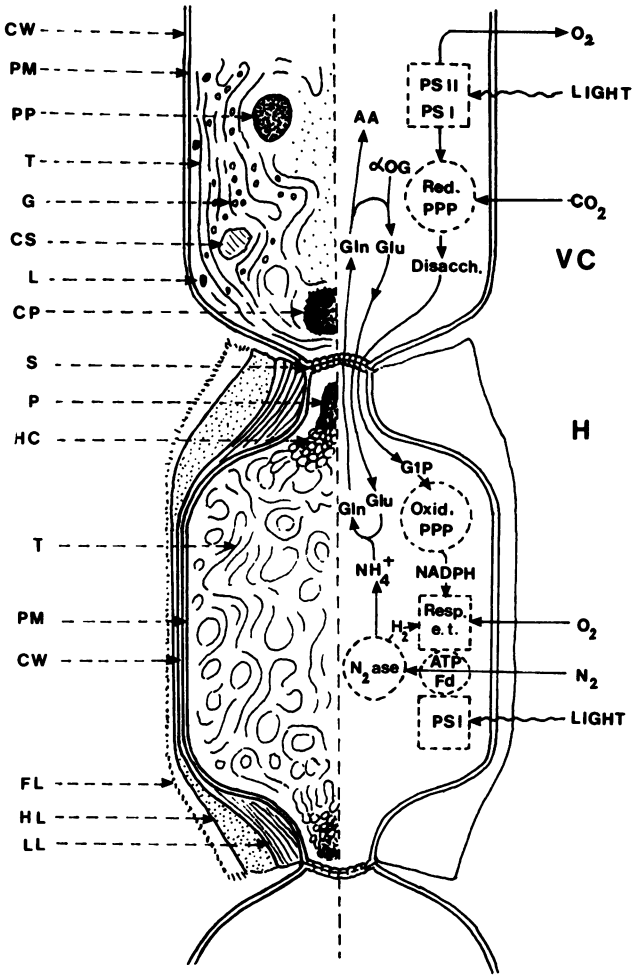

FIG. 5. Diagram illustrating the structural features and metabolic activities of a heterocyst (H) in relation to an adjacent vegetative cell (VC). Abbreviations: CW, cell wall; PM, plasma membrane; PP, polyphosphate body; T, thylakoid; G, glycogen granule; CS, carboxysome; L, lipid droplet; CP, cyanophycin granule; S, septum with microplasmodesmata; P, "plug"; HC, honeycomblike membranes; FL, HL, LL, fibrous, homogeneous, and laminated layer, respectively, of heterocyst envelope; AA, amino acids; Glu, glutamate; Gln, glutamine; Red. PPP, reductive pentose phosphate pathway; Oxid. PPP, oxidative pentose phosphate pathway; G1P, glucose phosphate; Fd, ferredoxin; Resp. e.t., respiratory electron transport.

greatly influenced by the prevalent O_2 tension under which heterocyst development is induced. The nitrogenase activity of cultures with heterocysts formed under air was very resistant to elevated pO_2 in the assay gas phase (223). Nitrogenase activity was more sensitive to O_2 in cultures in which heterocyst differentiation was induced under decreased O_2 tensions (355). Nitrogenase synthesized under anaerobic conditions was highly susceptible to inactivation, with exposure to atmospheric levels of O_2 causing total and irreversible destruction of the enzyme (272). However, cultures in which heterocyst formation was initiated under hyperbaric O_2 concentrations (200% of air saturation) required exogenous O_2 for maximum nitrogenase activity. From the correlation between O_2 tolerance and K_m for acetylene reduction, it was assumed that the development of a gas diffusion barrier into the heterocyst is controlled by the

TABLE 4. Effect of O_2 on $^{15}N_2$ fixation by a cell extract of *Chlorogloeopsis fritschii*[a]

Preparation[b]	Incubation[c]	^{15}N excess (atom%)
Aerobic	Aerobic	0.017
Anaerobic	Aerobic	0.022
Aerobic	Anaerobic	0.042
Anaerobic	Anaerobic	0.087

[a] Reprinted with permission from Fay and Cox (92).
[b] Disruption of cells in a French press at 4°C under (anaerobic) or without (aerobic) a continuous stream of argon.
[c] In the dark for 1 h under a gas phase of 10% N_2 (45.5% ^{15}N) with (aerobic) or without (anaerobic) 10% O_2 and the rest argon.

O_2 tension to which the culture is subjected during the period of heterocyst induction (223).

A mutant of the marine *Anabaena* sp. strain CA grew well aerobically in the presence, but not in the absence, of combined nitrogen in the medium (135). Its growth rate in a nitrogen-free medium was substantially increased under microaerobic incubation, implying that the ability of the mutant strain to protect its nitrogenase from damage by O_2 was impaired.

Planktonic *Anabaena* species seem to have developed adaptation responses to the diurnal changes in the dissolved-O_2 concentration in surface waters. In waters where concentrations of O_2 may reach 120% saturation, the capacity of mechanisms that protect nitrogenase against oxygen damage within heterocysts may become exhausted, and planktonic cyanobacteria appear to use additional means to overcome the effects of prolonged O_2 supersaturation. These may include changes in pigment (particularly phycobiliprotein) concentration, temporal separation of photosynthetic and nitrogenase activities (243), and the vertical migration of gas-vacuolated forms (268).

The pattern of heterocyst differentiation was found to be essentially similar when *A. cylindrica* was incubated in the light under either aerobic or microaerobic conditions (under a continuous stream of N_2-CO_2) following the transfer of filaments into a medium free from combined nitrogen (188). Not only did heterocyst formation continue under microaerobic incubation, but also both heterocyst production and nitrogenase synthesis were enhanced at low O_2 tensions.

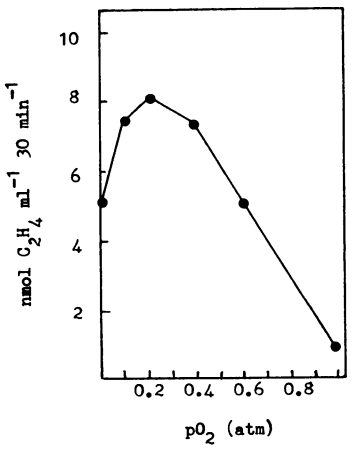

FIG. 6. Effect of pO_2 on nitrogenase activity (acetylene reduction) of *A. flos-aquae* after incubation in the light (3 klx) for 2 h. Adapted with permission from Stewart and Pearson (323).

TABLE 5. Effect of pO_2 on heterocyst development in *A. cylindrica*[a]

Event	Time (h) required under:		
	Hypobaric conditions[b]	Ambient O_2 tension[c]	Hyperbaric conditions[d]
Onset of heterocyst differentiation	27	24	15
Completion of first-generation heterocysts	47	39	26
Heterocyst development	20	15	11

[a] From Hatfull and Fay (148). Induction of heterocyst differentiation was effected by the transfer of filaments from medium containing NH_4^+-N into N-free medium, followed by incubation in the light (2 klx).
[b] 1% CO_2 in N_2.
[c] 1% CO_2 in air.
[d] 1% CO_2 plus 30% O_2, the rest N_2.

The differentiation of proheterocysts and their maturation into heterocysts were somewhat delayed under microaerobic treatment, but nitrogenase activity increased more rapidly. Under both aerobic and microaerobic conditions, enzyme activity was measurable only on completion of heterocyst development, notably when all three (fibrous, homogeneous, and laminated) envelope layers were deposited external to the cell wall.

When heterocyst differentiation was initiated under a range of O_2 tension, the influence of O_2 on heterocyst development was more evident (148). The onset of differentiation was most rapid under elevated pO_2 (0.3 atm [30.39 kPa]), and the rate of transformation was enhanced by the increase in O_2 concentration. Lipid accumulation at the cell periphery, concomitant with the formation of the lipidic laminated layer, was more abundant and the newly formed intracytoplasmic membrane system appeared more profuse under increased pO_2 (Table 5). The ultrastructure of vegetative cells appeared just the same whether filaments were incubated under atmospheric, hyperbaric, or hypobaric O_2 tensions, suggesting that there was no apparent structural adaptation in vegetative cells to changes in the concentration of external pO_2 or to a possible induction of nitrogenase synthesis in these cells under microaerobic incubation.

Rippka and Stanier (272) have devised a technique for a strictly anaerobic induction of heterocyst differentiation and nitrogenase synthesis by depressing PS II-dependent O_2 evolution with DCMU, in addition to incubating filaments under an anaerobic gas phase in the light. Nitrogenase synthesized under such conditions was shown to be extremely sensitive to O_2, which was attributed to the arrest of heterocyst development at the proheterocyst stage, prior to completion of the envelope. This assumption was based on electron-microscopic examination of heterocysts fixed with glutaraldehyde-osmium. However, the envelope layers, particularly the laminated layer, are not retained during preparation unless heterocysts are fixed with glutaraldehyde-permanganate (194, 362), a situation clearly indicated by the empty space in electron micrographs. An alternative explanation offered by the authors for the increased O_2 sensitivity of nitrogenase produced in the total absence of O_2 is that the development of certain mechanisms for the protection of nitrogenase against inactivation by O_2 is subject to induction by O_2. It is probable that the inhibition of photoevolution of O_2 by DCMU is a critical, or even more important, factor in depressing the induction of such mechanisms than the scavenging of O_2 from the external environment.

Structural Adaptations for Protection of Nitrogenase

Heterocyst envelope. During heterocyst differentiation, the three envelope layers are deposited in sequence (first the fibrous, then the homogeneous, and finally the laminated layer), outside the existing cell wall (Fig. 5). The observation that the formation of the laminated layer coincides with the appearance of nitrogenase activity suggests that the envelope may have an essential role in maintaining optimum conditions for N_2 fixation in the heterocyst (188). In an attempt to clarify this role, Wolk and coworkers have investigated and characterized the chemical nature of the envelope. Four unique glycolipids were isolated from the laminated layer of the envelope (362, 367). Glycolipids characteristic of heterocystous cyanobacteria were classified as hexose derivatives of long-chain polyhydroxyalcohols (33, 229). The homogeneous and fibrous layers were shown to be composed mainly of polysaccharide (42, 43, 73, 362). Both the laminated and homogeneous layers are thought to limit the rate of gas diffusion into the heterocyst (191, 226, 366). Boron and calcium deficiencies were reported to depress nitrogenase activity under aerobic, but not microaerobic, conditions, and it has been proposed that the elements may be required to stabilize the envelope structure (117, 277). The removal of calcium from the growth medium has been shown to increase heterocyst frequency (377), a possible symptom of inadequate N_2 fixation. It was also reported that heterocysts accumulate higher concentrations of calcium than vegetative cells do (377).

Mutants of *A. variabilis* strain ATCC 29413 deficient in envelope glycolipids were shown to lack or have very little nitrogenase activity when assayed under aerobic conditions (149). Revertants, which were able to fix N_2 in air, were shown to contain increased quantities of glycolipids. These observations support the view that the glycolipids of the laminated layer play an essential part in the protection of nitrogenase, possibly by restricting O_2 diffusion into the heterocyst. The ability of the heterocyst envelope to act as a gas diffusion barrier has also been indicated by the observation that the apparent K_m of nitrogenase for acetylene reduction was 10-fold higher in intact filaments than in cell extracts (142). No similar difference was observed in tests with the nonheterocystous *P. boryanum*. Jensen and Cox (169) demonstrated that the respiration rates of N_2-fixing cultures of *Anabaena* species displayed a biphasic dependence on pO_2 that seemed to correspond to the sum of two activities with different K_m values (1.0 and 69 μM, respectively). The high-affinity activity was observed only in cultures grown with nitrate and lacking heterocysts. Hence the low-affinity activity was attributed to O_2 uptake by heterocysts. This supports the idea that O_2 enters heterocysts much more slowly than it enters vegetative cells and that the heterocyst envelope acts as a significant barrier to the diffusion of O_2. The low-affinity activity was absent in mutant strains of *Anabaena* sp. strain PCC7120 that either lack the major glycoplipid of the laminated layer or have a defective homogeneous layer (226). Such deficiencies apparently reduce or eliminate the barrier to entry of O_2 into the heterocyst.

In view of the gaseous exchanges that accompany metabolic activity in the heterocyst, it is obvious that the heterocyst envelope cannot be impermeable to gases. In a subtle study on the permeability of heterocysts to N_2 and O_2, Walsby (350) measured the rate of gas diffusion into the gas-vacuolate vegetative cells and heterocysts of *A. flos-aquae* by determining the rate of gas pressure rise required

to collapse half of their gas vesicles. This critical rate was about 100 times lower for heterocysts than for vegetative cells. From the data obtained, it was possible to calculate that the mean gas permeability coefficient of heterocysts was 0.32/s for N_2 and 0.28/s for O_2. This is more than adequate to permit a rate of N_2 influx that will support the maximum rate of N_2 fixation and low enough to restrict the rate of O_2 intake to a level that can be reduced close to zero by the presumed rate of respiratory O_2 consumption, thereby maintaining anaerobic conditions in the heterocysts. In the dark, however, the rate of O_2 influx in the heterocysts may limit oxidative phosphorylation, and indirectly N_2 fixation, through the shortage of ATP.

Pore channel and septum. The envelope almost completely encompasses the heterocyst, apart from the minute (about 0.07-μm^2) area of contact between the heterocyst and the adjacent vegetative cell (194, 350) (Fig. 5). This is a remnant of the septum that originally separated the two vegetative cells. A narrow tubelike pore channel, formed by the enormous local expansion of the envelope layers, interconnects the heterocyst cytoplasm proper with the greatly reduced septum (279). The septum is traversed by ultrafine plasma bridges called microplasmodesmata (194), each about 8 nm in diameter and about 80 nm long (120). Their numbers are reduced during heterocyst differentiation by a factor of between 3 and 5. It is noteworthy that whereas only 30 to 40 microplasmodesmata traverse the septa between adjacent vegetative cells in nonheterocystous filamentous cyanobacteria, 100 to 250 such structures were recorded in the septa between adjacent vegetative cells of heterocystous cyanobacteria (122). This may indicate the requirement for a higher-capacity transport system in the filaments of heterocystous cyanobacteria.

According to calculations by Walsby (350), the maximum permeability coefficient of heterocyst pores is about 0.0043/s, which is about 75 times less than the permeability of the envelope. It is therefore unlikely that the terminal pores constitute important gas diffusion pathways. They are thought, however, to function in the exchange of metabolic products and are probably the main routes of intercellular solute transport (145). It is therefore reasonable to assume that the junction between the heterocyst and vegetative cells constitutes a complex and highly regulated area of contact between the O_2-evolving vegetative cells and the anaerobic environment of the heterocyst.

Cell membrane and intracytoplasmic membrane system. The plasma membrane was formerly thought to remain unchanged during heterocyst differentiation (130), but more detailed studies by Giddings and Staehelin (120), using freeze-fracture electron microscopy, have revealed differences in the size and quantity of particles embedded in the plasma membranes of the two cell types. The density of particles on the protoplasmic fracture (PF) face decreased during heterocyst differentiation from an average of 2,236 to 1,724/μm^2, whereas the density of particles on the exoplasmic fracture (EF) face remained unchanged at about 550/μm^2. The mean sizes of particles on the PF face of the plasma membrane of vegetative cells and heterocysts are similar (6.65 nm). However, the mean particle size is larger (8 nm) on the EF face of the heterocyst cell membrane than on the EF face of the vegetative cell membrane (6.8 nm). Furthermore, a histogram illustrating the size distribution of particles on the EF face of the vegetative cell membrane is biphasic, with peaks at 5.5 and 7.5 nm, whereas the histogram for the heterocyst EF face particles displays only a single peak at 7.5 nm. These observations were thought to imply that one or more proteins present in the vegetative cell membrane are absent in the plasma membrane of heterocysts. Biochemical evidence for this and for the possible significance of these differences is lacking.

Profound changes in the nature and distribution of the intracytoplasmic membrane system (photosynthetic membranes or thylakoids) occur during heterocyst development. The typically peripheral and concentric membranes of the vegetative cell disintegrate, and a new confluent and elaborate membrane system that is distributed more evenly throughout the heterocyst cytoplasm is formed (193, 194, 359). In the course of heterocyst maturation, the membranes become increasingly contorted and reticulate, particularly near the polar region, where they acquire a latticelike or honeycomblike configuration. Concomitant with the breakdown and new formation of the intracytoplasmic membrane system, the biochemical machinery of the differentiating cell undergoes extensive changes (discussed below). It has been postulated that elaboration of the membrane system is essential for providing the mature heterocyst with the requirements of reducing power and ATP for N_2 fixation (188).

Freeze-fracture replica studies have also revealed the differences in the architecture and composition of the photosynthetic membranes of the two cell types (121). Whereas the EF face of vegetative cell thylakoid membranes displays particles 7.5 and 10 nm in size, heterocyst membranes lack the 10-nm particles and contain particles mainly of 5.5 nm in size and some of 7.5 nm in diameter. The density of particles on the EF face of thylakoid membranes is slightly decreased in heterocysts (715/μm^2, as compared with 940/μm^2 in vegetative cells). Particles on the PF faces of vegetative cell and heterocyst thylakoid membranes show a less distinct distribution, with a mean diameter of 7.1 nm for vegetative cells and 6.4 nm for heterocysts; the density of particles over the membrane face appears to be similar (about 5,600/μm^2) in the two types of cell.

Giddings and Staehelin (121) have proposed that the 10-nm particles on the EF face of vegetative cell thylakoid membranes, which are absent in heterocysts, may correspond to PS II complexes. Speculating on the identity of the 5.5-nm particles, seen only on the EF faces of heterocyst thylakoid membranes, they thought that these may represent the nitrogenase complex. Although immunocytochemical studies on the localization of nitrogenase suggest an even distribution within the heterocyst cytoplasm of the enzyme complex (similar to the distribution of thylakoid membranes), they provide no conclusive evidence for or against a more direct association of nitrogenase with the thylakoid membranes (18) (see below).

Biochemical Changes Promoting Protection and Activity of Nitrogenase

When filaments of heterocystous cyanobacteria are deprived of combined nitrogen, a rapid turnover of vegetative cell protein occurs (96, 97). Protein degradation is affected by the activation of proteolytic enzymes (369), which causes the breakdown of phycobiliprotein pigments in all vegetative cells (227) and the dissolution of enzymes associated with photosynthetic CO_2 fixation in developing heterocysts (55, 361). It has been shown that 17 of the 35 major proteins present in vegetative cells are absent in the heterocysts and that the majority of these are degraded during the early stages of heterocyst differentiation (97, 369). The synthesis of polypeptides and proteins characteristic of heterocysts, including the structural proteins of nitrogenase, begins long

before nitrogenase activity is detectable (97). Protein turnover results in a fundamental reorganization of the biochemical machinery of the differentiating cell, in an apparent adaptation to the promotion and protection of nitrogen-fixing activity in mature heterocysts.

Transformation of photochemical apparatus. Heterocysts remain pigmented after their formation, but appear generally paler than vegetative cells. The faint appearance of heterocysts under the light microscope suggests that pigment composition has been modified during differentiation and that both the organization of the photosynthetic apparatus and the photochemical activities of the heterocysts have been altered. Early studies have shown that heterocysts contain significantly reduced quantities of photosynthetic pigments, particularly phycobiliproteins (86, 338, 367), and that they have lost the ability to fix CO_2 in the light (95, 320, 363) and to evolve O_2 when illuminated (29, 48, 334). The changes were thought to indicate that heterocysts lack a functional PS II and the reductive pentose phosphate pathway. These assumptions were supported by the demonstration that isolated heterocysts fail to perform the Hill reaction; have a reduced yield of chlorophyll *a* fluorescence and no light-induced changes in fluorescence yield (69); are deficient in manganase (334), an essential component of the O_2-evolving mechanism; and lack enzymes of the reductive pentose phosphate pathway (319, 361). On the other hand, demonstration of light-dependent electron transport (165, 367), of a PS I-type action spectrum of nitrogenase activity (87), of light-induced oxidation of reaction center chlorophyll P700 (69), and of photosynthetic phosphorylation in isolated heterocysts (289, 325), strongly argues for the retention of an active PS I in heterocysts.

It is well documented (124) that, in cyanobacteria, phycobiliprotein pigments are associated primarily with PS II, actively transferring light energy to chlorophyll *a*. Phycobiliprotein concentrations in heterocysts seem to vary with the age of the culture and the conditions of growth (255, 339). Yamanaka and Glazer (372) have shown that the phycobiliprotein pigments present in mature heterocysts were not newly synthesized in the course of heterocyst differentiation or thereafter, but represent the residue of pigments originally present in the vegetative cell prior to differentiation. When heterocyst development is initiated by the transfer of filaments from a medium containing combined nitrogen to one deficient in nitrogen, a state of nitrogen starvation is induced (63). Cells respond to this by mobilization of nitrogenous storage products, such as cyanophycin granules (296) and phycobiliproteins, organized in well-defined particles called phycobilisomes (124). These structures are degraded in all vegetative cells by the action of proteolytic enzymes (188, 227, 369). A proportion of the nitrogen-depleted cells develop into heterocysts. Such "first-generation" heterocysts contain only residual levels of biliprotein pigments, namely the remainder of the structural units that build the phycobilisome particles. Once N_2 fixation begins in the initial heterocysts, fixed nitrogen is transferred to the vegetative cells, allowing cell and filament growth. Growth and cell division lead to the formation of new heterocysts from vegetative cells that are then relatively rich in biliproteins. Phycobilisomes in these heterocysts, although modified to a lesser extent, remain active in light absorption and energy transfer to chlorophyll in PS I (255, 372). It is therefore unlikely that changes in the concentration and organization of phycobiliprotein pigments could be the principal or sole reason for the absence of PS II activity in the heterocyst. Equally important seems to be the marked reduction in the concentrations of light-harvesting chlorophyll *a* and possibly of chlorophyll-protein complexes (3), as well as of cytochrome *b-559* (5), associated with PS II, in addition to the greatly reduced levels of bound manganese (334). The consequent cessation of photosynthetic O_2 evolution in the developing heterocyst is no doubt one of the main preconditions for the expression of *nif* genes in the mature heterocyst (see below).

TABLE 6. Content of chlorophyll and size and relative numbers of photosynthetic units (PSU) in vegetative cells and heterocysts of cyanobacteria[a]

Cell	Chlorophyll content (10^{-7} μg/cell)	PSU size[b]	PSU/cell (10^5 P700 molecules/cell)
Vegetative cells			
Nostoc sp.	1.4	180	2.6
Anabaena sp.	1.7	180	3.1
Heterocysts			
Nostoc sp.	0.7	60	4.0
Anabaena sp.	0.9	60	5.0

[a] Adapted with permission from Alberte et al. (3).
[b] Ratio of total chlorophyll to P700 (the reaction center of PSI).

There is a great deal of additional evidence in support of the presence of an active PS I in the heterocysts. Analysis of thylakoid membranes from isolated heterocysts has revealed that components of the photosynthetic electron transport chain (i.e., cytochrome *c-554*, plastocyanin, plastoquinone, P700, cytochrome *b-563*, and F-S proteins) are present and functional in heterocysts (336). Isolated heterocysts have been shown to perform photooxidation and photoreduction of reaction center P700, photosynthetic electron transport, and photophosphorylation. Heterocysts contain ferredoxin and ferredoxin:$NADP^+$ oxidoreductase in adequate quantities to affect the photoreduction of $NADP^+$ (335). It has also been shown that, as a result of reorganization of the thylakoid membranes, the size of photosynthetic units becomes greatly reduced in the heterocysts compared with that in vegetative cells. This allows the packaging of about 35% more photosynthetic units within the thylakoid membranes of heterocysts (3) (Table 6). Consequently, heterocysts have an increased content of PS I, capable of meeting the augmented requirements of the N_2-fixing process.

Among the cytoplasmic inclusions that disintegrate during heterocyst formation are the paracrystalline carboxysomes, composed of Rubisco. In vegetative cells, carboxysomes contain the bulk of Rubisco (53), and it is possible, although not proven, that they represent the active form of the enzyme (19, 52, 264). The degradation of these structures during heterocyst differentiation tallies well with the lack of CO_2-fixing activity in heterocysts (54, 319, 361). Transcripts for the *rbcL* and *rbcS* genes, encoding the large and small subunits of Rubisco, respectively, were shown to be absent in heterocysts of *Anabaena* sp. strain PCC7120 (129). The inevitable consequence of the absence of PS II and of enzymes of the reductive pentose phosphate pathway in heterocysts is that the carbon requirement of heterocysts must be provided by the vegetative cells, probably in the form of carbohydrate. The carbon transported serves as a source of reductant for N_2 fixation and as a substrate for respiratory energy metabolism.

Synthesis of nitrogenase and appearance of nitrogenase activity. When *Anabaena* filaments are transferred from a nitrogen-containing medium to a nitrogen-free medium, a lag

of about 20 h occurs before the initial stages of heterocyst differentiation can be observed by light or electron microscopy (188, 227). Nitrogenase activity, however, becomes manifest only a few hours later, when the transformation of the first heterocysts is completed.

Fleming and Haselkorn (96, 97) have used polyacrylamide gel electrophoresis, combined with ^{35}S labeling, to determine the changes in protein composition of *Anabaena* vegetative cells and heterocysts following transfer of filaments to a nitrogen-free medium. They noticed a rapid turnover of vegetative cell protein, as a result of high proteolytic activity early in heterocyst development. The synthesis of several proteins, including the structural proteins of nitrogenase, was thought to be initiated in all cells of the filament and then to proceed only in the developing proheterocysts. This assertion, however, was found to be incorrect after the subsequent recognition that nitrogenase cannot be synthesized until the *nifHDK* genes are rearranged during a later stage of heterocyst development (127, 128; see below). Hence nitrogenase proteins are synthesized exclusively in the heterocysts during the initial process of differentiation, as in the already differentiated filaments (96).

Studies on the localization of nitrogenase in a variety of heterocystous species by means of immunoelectron microscopy have provided further evidence of the presence of nitrogenase only in heterocysts. Labeling was not associated preferentially with the thylakoid membranes or with any other particular structure or regions of the heterocyst, but was evenly distributed throughout the heterocyst cytoplasm (7, 18). A similar pattern of labeling was observed in endosymbiotic cyanobacteria from the lichens *Peltigera aphthosa* and *Nephroma arcticum* (19), from the bryophyte *Anthoceros* (266) (Fig. 7), from the fern *Azolla* (30), from the coralloid roots of *Cycas revoluta* (18), and in the cells of the angiosperm *Gunnera* (308). It is noteworthy that there was no indication of the presence of nitrogenase in vegetative cells of the cyanobionts from lichens, despite earlier evidence of greatly reduced O_2 tensions within the lichen thalli (212).

The increased O_2 sensitivity of nitrogenase observed when heterocystous cyanobacteria were incubated under microaerobic or anaerobic conditions, following transfer to a nitrogen-free medium (272, 343), has been attributed to enzyme synthesis in vegetative cells, which are devoid of mechanisms for the protection of nitrogenase against damage by O_2. As mentioned above, this sensitivity can be equally explained by the assumption that the expression of genes coding for the development of protective mechanisms in heterocysts is O_2 dependent. In an attempt to resolve the controversial problem of nitrogenase synthesis in vegetative cells of heterocystous cyanobacteria grown in the absence of O_2 (188, 272, 305, 343, 354), Murry et al. (223) induced nitrogenase synthesis in cultures of *A. cylindrica* after transfer of filaments to a nitrogen-free medium and incubation under strictly anaerobic conditions in the presence of DCMU and under 100% argon. They then applied immunoferritin labeling to localize the Mo-Fe-protein of nitrogenase and demonstrated that significant labeling was present only in the heterocysts. From this they concluded that nitrogenase synthesis is restricted to heterocysts in heterocyst-forming cyanobacteria, even in the complete absence of exogenous and endogenous sources of O_2, thus confirming earlier observations (148, 188, 354).

A similar conclusion has been reached recently by Elhai and Wolk (79) from results of a study in which the *nif* structural genes (*nifHDK*) have been monitored by fusing the *nifHDK* promoter to *luxAB*, encoding luciferase, and transferring the resulting plasmid into *Anabaena* sp. strain PCC7120 (Table 7). Light emission was used to localize the transcription of *nif* genes. The expression of the *nif* promoter was confined to heterocysts or partially differentiated heterocysts during both aerobic and anaerobic induction of nitrogenase synthesis.

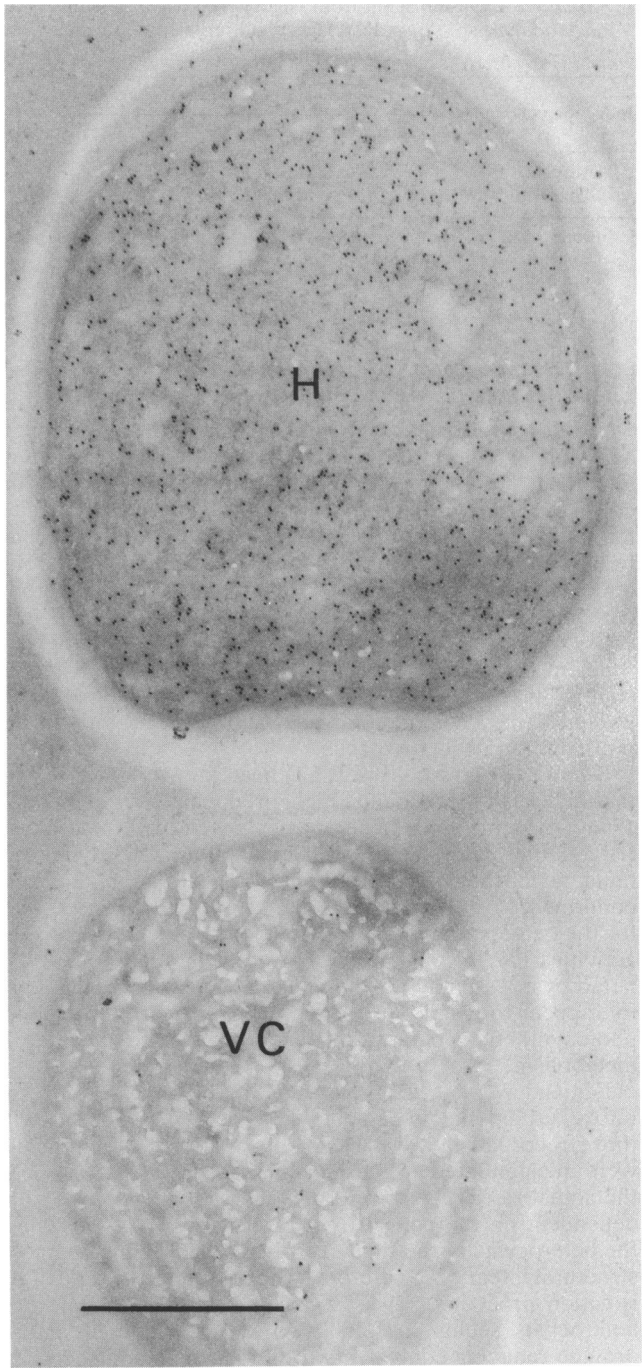

FIG. 7. Immunogold localization of the Fe-protein of nitrogenase in a *Nostoc* heterocyst isolated from the bryophyte *Anthoceros punctatus*. Abbreviations: H, heterocyst; VC, vegetative cell. Bar, 1 μm. Courtesy of B. Bergman and A. N. Rai.

Artificially produced mutants of the same *Anabaena* spe-

TABLE 7. Light emission from vegetative cells and heterocysts of *Anabaena* sp. strain PCC7118 resulting from expression of P *nifHDK*[a]-*luxAB*[b,c]

Induction conditions	Mean light flux density (counts/min/μm²)		
	Background	Vegetative cells	Heterocysts
Aerobic	0.24	0.30	3.9
Anaerobic	0.21	0.30	5.1

[a] Promoter of *nifHDK* operon.
[b] Genes encoding luciferase.
[c] Reprinted with permission from Elhai and Wolk (79).

TABLE 8. Effect of azide on nitrogenase activity and on Fe-Mo-protein (component 1) of *A. cylindrica* nitrogenase after a 3-h treatment of log phase cultures[a]

Growth conditions	NaN_3 (20 mM) addition	Acetylene reduction (% of time zero level)	Fe-Mo-protein (% of time zero level)
Aerobic[b]	No	100	97
	Yes	0.3	23 (75% loss)
Microaerophilic[c]	No	100	103
	Yes	42.0	67 (35% loss)

[a] Reprinted with permission from Murry et al. (223).
[b] 0.3% CO_2 in air.
[c] 0.3% CO_2 in N_2.

cies, which either formed morphologically abnormal heterocysts or were apparently unable to differentiate heterocysts, were shown to reduce acetylene only under microaerobic or anaerobic conditions or failed to display nitrogenase activity under any conditions (35, 309). These observations are consistent with the view that one of the prime prerequisites for the expression of *nif* genes in heterocystous cyanobacteria is the completion of heterocyst development, not only under aerobic but also under microaerobic and anaerobic conditions. This concept is also compatible with ecological considerations, since O_2 concentrations in the natural environment often fluctuate rapidly and considerably. The substantial investment into nitrogenase synthesis would be wasted if the enzyme complex was produced without due protection in vegetative cells.

Respiratory metabolism. It has been known for some time that heterocysts maintain a highly reducing environment. In 1956 Drawert and Tischer (70) showed that heterocysts reduce the redox indicator triphenyltetrazolium chloride much faster than vegetative cells do. Their finding has been confirmed by others, using a variety of cytochemical tests for the detection of dehydrogenase and cytochrome oxidase activities (93, 320, 330). Strong reducing conditions in the heterocyst, essential for the maintenance of N_2-fixing activity, appear to result partly from the absence of O_2-evolving photosynthetic apparatus and also from elevated respiratory metabolism.

Fay and Walsby (95) measured 40% higher rates of respiratory O_2 uptake in isolated heterocysts compared with those recorded in whole filaments. Although their results were substantiated in subsequent investigations (29, 253), the actual rates of respiratory O_2 uptake were shown to be dependent on the pool of respiratory substrates present in the heterocysts at the time of isolation and on the degree of structural integrity of heterocysts recovered at the end of the isolation procedure (194, 228). Respiratory metabolism in heterocysts seems to fulfill two main functions, first to provide reducing equivalents to nitrogenase, and second to protect the enzyme system from inactivation by O_2 by maintaining an anaerobic microenvironment at the site of enzyme function.

As a consequence of the loss of CO_2-fixing ability, metabolic activities in heterocysts must rely entirely on the import of carbon from the adjacent vegetative cells. N_2-fixing activity and respiratory protection of nitrogenase require a continuous flow of carbon (363) and possibly a certain degree of accumulation of carbon reserves in the heterocysts. The latter is indicated by the observation that glycogen-containing granules and lipid droplets increase in number within heterocysts when N_2 fixation is prevented by interception of the supply of N_2 to the culture (63, 89, 190). The importance of an adequate flow of carbon into heterocysts is corroborated by the findings that nitrogenase activity is closely related to the size of the reductant pool (84, 88, 199) and that the O_2 sensitivity of nitrogenase is markedly increased in stationary and reductant-depleted cultures (223, 355). The identity of the imported compound(s) remains uncertain (2, 364), although many workers presume that disaccharide(s) is the most probable source of reductant (173, 265). Disaccharides can be converted readily to the component sugars in the heterocysts, degraded along the glycolytic pathway, and oxidized through the hexose monophosphate shunt (oxidative pentose phosphate pathway), providing energy and reductant for N_2 fixation (Fig. 5). There is a great deal of evidence that the catabolic pathways remain operational after heterocyst differentiation. The presence of enzymes involved in these pathways was first reported by Winkenbach and Wolk (361). This has been confirmed and the list of the enzymes involved has been completed in subsequent investigations (197, 200, 228, 253). The specific activity of glucose-6-phosphate dehydrogenase was shown to be particularly high (6, 197, 228). Although this enzyme is known to be inhibited by ribulose 1,5-bisphosphate in photosynthesizing cells (249), it remains active and light independent in heterocysts (6). Added key intermediates of the oxidative pentose phosphate pathway (glucose 6-phosphate, fructose 6-phosphate, and 6-phosphogluconate) were found to enhance O_2 uptake considerably in isolated heterocysts (253). It has also been shown that Krebs cycle enzymes are present in isolated heterocysts and that the cycle remains incomplete, as in the vegetative cells (228). Intermediates of the cycle had the effect of enhancing nitrogenase activity when they were added to isolated heterocysts (200, 265, 288). They do not appear, however, to represent an important source of reductant for nitrogenase in the light or to contribute significantly to respiratory O_2 protection of the enzyme complex (223).

The uptake of O_2 by isolated heterocysts with added organic compounds or metabolic intermediates is enhanced by the presence of $NADP^+$. Heterocysts have also been shown to possess NADPH-oxidase activity (253), as well as an active electron transport system that can transfer electrons from glucose 6-phosphate to ferredoxin (6, 25). The role of respiratory electron transport, particularly that of the cytochrome system in mopping up O_2 from the site of nitrogenase activity, thereby protecting nitrogenase against inactivation by O_2, is evident from the observation that inhibition of cytochrome oxidase activity led to a substantial loss of the Fe-Mo-protein of nitrogenase during aerobic incubation of *A. cylindrica* (223) (Table 8).

The occurrence of light-dependent O_2 consumption by

heterocysts (29, 301) may be of particular importance under natural conditions. The rate of such O_2 uptake was reported to be three times higher in heterocysts of *A. cylindrica* than in vegetative cells and to increase with increasing irradiance (up to 2.5 mW cm^{-2}) to a level five times higher than that which saturates photosynthetic O_2 production. It has been suggested that light-driven O_2 consumption is coupled to pseudocyclic electron flow in PS I and that the reduction of O_2 is at the expense of reduced carbon in the heterocyst (301). This assumption was based on the observation that depletion of carbon reserves during preincubation in the dark caused a decline in light-dependent O_2 consumption, as well as in the rate of endogenous respiration in the dark. Further investigations are required to assess the role of light-dependent O_2 consumption in the protection of nitrogenase.

The elevated rates of O_2 consumption, the increased activities of enzymes of the catabolic pathways, and the formation of a greatly extended cytoplasmic membrane system are consistent with the conclusion that high respiratory activity in heterocysts is probably the principal mechanism of O_2 protection. The cytoplasmic membranes are thought to incorporate both the photosynthetic and respiratory electron transport systems of the heterocyst (164, 251).

Oxyhydrogen reaction. Heterocystous cyanobacteria possess two distinct hydrogenases with different localities and functions. One of these is the classical reversible hydrogenase which is present in soluble form in both vegetative cells and heterocysts. It enables the organism to utilize exogenous H_2 as a source of reductant during growth in an O_2-free environment (163). The other is the unidirectional or uptake hydrogenase, induced by H_2 (27, 332), which is membrane bound and confined to the heterocysts (161, 170, 257). This enzyme catalyzes the oxyhydrogen (or Knallgas) reaction, associated with O_2 reduction. Its function, as in other diazotrophs, is the recapture of H_2 released during the nitrogenase reaction (254). Both H_2 evolution by nitrogenase and H_2 consumption by uptake hydrogenase proceed concurrently during active growth on N_2 (162). The occurrence in heterocystous cyanobacteria of nitrogenase-catalyzed H_2 evolution, uptake hydrogenase-catalyzed H_2 consumption, and H_2-supported nitrogenase activity was first reported by Benemann and Weare (12, 13). Their findings were confirmed and the conditions and possible functions of the oxyhydrogen reaction were studied in more detail in subsequent investigations (26, 59, 82, 172, 254, 257, 332, 333).

The initial problems of cellular localization of the two hydrogenases in heterocystous cyanobacteria (78, 257, 333) have largely been resolved by the application of selective assay methods. Investigations by Houchins and Burris (161) have established that the reversible hydrogenase is independent of N_2-fixing activity and that the uptake hydrogenase is limited to heterocysts of aerobically grown filaments of *Anabaena* sp. strain PCC7120. A small amount of activity (about 1% of that present in heterocysts) was found in a vegetative cell preparation from cultures grown under microaerobic conditions (161). It is difficult to ascertain whether this barely significant activity corresponded to a limited induction of the enzyme in vegetative cells or to the presence of incipient heterocysts in the vegetative cell preparation. These are usually undetectable under the light microscope. The confinement of both nitrogenase and hydrogenase activities to the heterocysts lends strong support to the hypothesis that the specific function of uptake hydrogenase is to recycle H_2 lost in the nitrogenase reaction. The uptake hydrogenase-catalyzed oxyhydrogen reaction in the heterocysts is mediated by the respiratory electron transport chain, terminating with cytochrome oxidase (26, 254). The reaction in which H_2 is used as a respiratory substrate is coupled to oxidative phosphorylation, thus providing an additional source of ATP for N_2 fixation. Nitrogenase activity is greatly enhanced under an atmosphere of H_2, and the activity is strictly light dependent (78, 189, 254).

TABLE 9. H_2 uptake by isolated heterocysts of *Anabaena* sp. strain PCC7120 in the dark and in the light (600 W m^{-2})[a]

Acceptor	E^0 (V)	Rate of H_2 uptake (nmol/μg of Chl[b]/h) in:	
		Dark	Light
None		0.04	0.07
O_2 (40 μM)	+0.82	3.2	3.2
Benzoquinone (1 mM)	+0.29	3.5	3.5
1,2-Naphthoquinone (1 mM)	+0.14	2.4	2.3
Phenazine methosulfate (1 mM)	+0.08	3.4	3.1
Menadine (1 mM)	−0.01	1.6	1.6
2-Hydroxynaphthoquinone (1 mM)	−0.14	0.09	0.84
Anthraquinone 2,6-disulfonate (1 mM)	−0.18	0.08	0.55
Anthraquinone 2-sulfonate (1 mM)	−0.22	0.06	0.56
NADP (1 mM)	−0.32	0.04	0.14
Methyl viologen (10 mM)	−0.44	0.05	0.17

[a] Reprinted with permission from Houchins and Burris (162).
[b] Chl, chlorophyll.

Whether stimulation of nitrogenase activity by H_2 in the dark is due solely to an additional supply of ATP generated in the oxyhydrogen reaction or is also attributable to direct donation of electrons to nitrogenase is a matter of controversy (12, 254, 368). The main argument against the concept of direct involvement of H_2 in the nitrogenase reaction is the finding that hydrogenase is incapable of reducing low-potential electron acceptors such as $NADP^+$ or ferredoxin in the dark and hence H_2 may be unavailable as a source of electrons for N_2 fixation (162) (Table 9). In the light, however, electrons could be rendered more energetic by an additional input of energy during photosynthetic electron transport in the heterocyst (26, 78, 162). An alternative interpretation of the light-stimulated, H_2-supported nitrogenase activity is to assume that there is combined action by cyclic photophosphorylation, providing ATP, and by a light-independent oxyhydrogen reaction, supplying electrons to nitrogenase, without the involvement of the photosynthetic electron transport chain (22). It has also been suggested that the two pathways of H_2 assimilation, the one catalyzed by hydrogenase with O_2 as electron acceptor and the other via nitrogenase donating electrons directly to N_2, may alternate, depending on the prevailing light conditions (357).

The removal of O_2 from the site of nitrogenase activity was considered to be one of the main functions of uptake hydrogenase in aerobic N_2-fixing organisms, thereby augmenting the effects of respiratory protection (68). Transfer of electrons to O_2 via the respiratory electron transport chain during the oxyhydrogen reaction should contribute to the maintenance of a microaerobic environment inside the heterocysts (26). Experimental results, however, seem to indicate that this contribution may be of no great importance (357) when compared with that of respiratory O_2 consumption and is insufficient on its own to protect nitrogenase from inactivation by hyperbaric O_2 concentrations (302).

Antioxidant enzymes and compounds. The occurrence of enzymes such as ascorbate peroxidase, superoxide dismu-

tase, catalase, and glutathione reductase, which are involved in the elimination of reactive oxygen species, has been demonstrated with a number of heterocystous cyanobacteria. Our knowledge regarding their activity in vegetative cells and heterocysts, particularly with respect to the protection of nitrogenase from active oxygen species, is, however, only rudimentary.

The presence of ascorbic acid in heterocysts of *A. cylindrica* was demonstrated first by Talpasayi (330); this was followed by reports of high concentrations of ascorbate and elevated ascorbic acid oxidase activity in extracts from N_2-fixing filaments of *A. ambigua* (347). The same authors located strong peroxidase activity in heterocysts by using cytochemical tests with benzidine nitroprusside. Significant activities of both ascorbate oxidase and catalase were measured in vegetative cells and heterocysts of *Nostoc muscorum* by Tel-Or and associates (331). The specific activity of catalase was shown to be particularly high in heterocysts. Considering, however, that the affinity of ascorbate peroxidase for hydrogen peroxide is much higher than that of catalase, ascorbate peroxidase may be much more effective in protecting the cells from the toxic effects of hydrogen peroxide. Ascorbate peroxidase activity could be limited by low substrate concentration in vegetative cells but not in heterocysts, which maintain much higher concentrations of ascorbate (347).

Superoxide anion radicals are apparently produced in both vegetative cells and heterocysts. Superoxide dismutase, which catalyzes the reduction of superoxide radicals, has been shown to be present in both cell types, although there is some discrepancy regarding enzyme concentrations (59, 134, 153). In view of the finding that the induction of superoxide dismutase is O_2 dependent (105, 202), it would be reasonable to assume that a relatively low enzyme activity in heterocysts is a reflection of the microaerobic environment in the N_2-fixing cells. Accordingly, superoxide dismutase in the heterocysts would have a limited but specific function in preventing the accumulation of superoxide radicals produced in the course of O_2 reduction. Glutathione reductase activity has been detected in the heterocysts and vegetative cells of *N. muscorum* (177). The enzyme may contribute to the removal of peroxide ions produced by the action of superoxide dismutase.

Light-independent oxidation of diaminobenzidine, a reaction characteristic of hemoproteins, has been found to be associated with the honeycomblike membranes and the periphery of heterocysts of *A. cylindrica* and to be enhanced in the presence of hydrogen peroxide (225). The reaction seems to imply the presence of a so far unidentified heme protein that could be involved in the degradation of hydrogen peroxide at certain critical regions of the heterocyst structure. Boron deficiency was reported to inhibit nitrogenase activity while increasing the activity of superoxide dismutase, catalase, and peroxidase in *Anabaena* sp. strain PCC7119, supporting the assumption that boron deficiency causes increased O_2 diffusion into heterocysts (117). It appears that the various enzymes involved in the elimination of toxic oxygen species may operate in a complementary fashion, whose nature and regulation are poorly understood.

De novo enzyme synthesis and conformational protection. The structural and biochemical properties of heterocysts discussed in the previous sections appear to provide adequate protection to nitrogenase against oxygen injury under normal atmospheric conditions. Transient or more prolonged exposure of nitrogenase to higher than atmospheric O_2 tensions, however, does seem to occur in nature, in surface waters or during periods of intense photosynthetic activity (243). Even ambient concentrations of O_2 could damage nitrogenase when protective mechanisms fail to operate in the event of a shortage of respiratory substrates and reductants (223, 355). It has been demonstrated in a number of studies that heterocystous cyanobacteria are able to regain most or part of their nitrogenase activity within a relatively short period (a few hours) after inactivation brought about by exposure to hyperbaric O_2 tensions (24, 258, 323). Whether such recovery is due to de novo nitrogenase synthesis, to some reversible change in the conformation of nitrogenase proteins, or both, is unclear.

In an attempt to explain the steady-state levels of nitrogenase during growth of *A. flos-aquae* under aerobic conditions, Bone (24) investigated the nature of recovery of nitrogenase after inactivation with 100% O_2. He found that the recovery of enzyme activity was inhibited up to 88% in the presence of inhibitors of protein synthesis, and he therefore attributed the recovery to new enzyme synthesis rather than to a reversible conformational change. His concept of "enzyme synthesis counteracting inactivation" has gained support in subsequent investigations by Benemann and colleagues (222, 224, 355). In a time course experiment with *A. cylindrica* incubated under a range of pO_2 values and in the presence of various inhibitors of protein synthesis, nitrogenase activity and Mo-Fe-protein were shown to be lost at a rate directly related to the pO_2 applied (224). Nitrogenase activity decayed faster than Mo-Fe-protein, and the decay of the protein was pO_2 dependent. Results were thought to indicate that O_2 inactivation of nitrogenase is followed by protein turnover and that the organism is able to alter the rate of nitrogenase synthesis to compensate for the rate of O_2-affected enzyme inactivation. It was proposed that nitrogenase is continuously inactivated by O_2 and degraded and resynthesized during normal growth of heterocystous cyanobacteria under aerobic conditions. Initial recovery of enzyme activity after O_2 treatment was found to be chloramphenicol insensitive; this was considered to possibly reflect the operation of conformational protection. Further recovery of both enzyme activity and Mo-Fe-protein was, however, inhibited by chloramphenicol, and this was thought to imply new synthesis of nitrogenase (222).

Studies with the marine *Anabaena* isolate, strain CA, by Tabita and associates also support the assumption that some kind of conformational protection of nitrogenase and new enzyme synthesis may not necessarily be exclusive adaptations of heterocystous cyanobacteria to aerobic N_2 fixation. Nitrogenase activity of this strain declined sharply on treatment with 100% O_2, and it was shown that the decline was due to inactivation of both nitrogenase components (258). Activity was restored in a process that required protein synthesis but not de novo synthesis of nitrogenase. Recovery was rapid when filaments were transferred to an argon atmosphere, although it also occurred during prolonged incubation under 100% O_2 (Fig. 8). From observations that (i) the recovery was more rapid than nitrogenase synthesis, (ii) the O_2 sensitivity of nitrogenase decreased on treatment of filaments with 100% O_2, and (iii) filaments also regained nitrogenase activity when synthesis was repressed by the addition of ammonia or nitrate, the investigators concluded that some kind of conformational mechanism, as well as new-enzyme synthesis, operates in this organism.

Subsequent studies have shown that treatment with 100% O_2 induces a reversible modification of the Fe-protein of nitrogenase, recognizable by a shift (slowdown) in the migration of protein during polyacrylamide gel electrophoresis

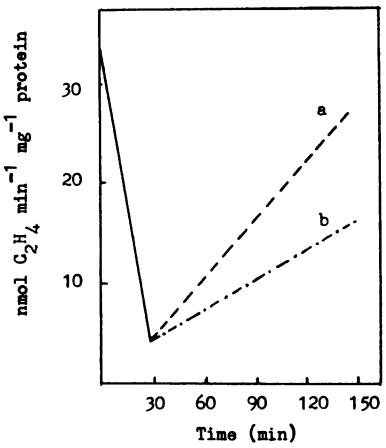

FIG. 8. O_2 inhibition and recovery of nitrogenase activity in *Anabaena* sp. strain CA. After exposure of a culture to 100% O_2 in the light for 30 min, suspension samples were transferred for a further 120 min of incubation under either 100% Ar (a) or 100% O_2 (b). Gas samples were withdrawn at intervals for acetylene reduction assay. Reprinted with permission from Pienkos et al. (258).

and corresponding to a change in molecular mass from 36 to 38 kDa (303, 329) (Fig. 9). Under conditions of low pO_2, the Fe protein was found in the 36-kDa form, whereas under atmospheric and hyperbaric levels of O_2, the altered 38-kDa modification was present. Since chloramphenicol did not affect the modification in either direction, it was assumed that the 38-kDa Fe-protein is required for enzyme function under ambient atmospheric conditions, because this form is protected from any deleterious effects of O_2. A similar modification of both Fe-protein subunits was demonstrated with *A. variabilis* on exposure of filaments to 100% O_2 (267) or as a consequence of carbon starvation (effected by controlling CO_2 fixation) (84). The latter reduces the supply of reductant to heterocysts and impedes respiratory O_2 protection of nitrogenase. Reversible modification of the Fe-protein also occurred during incubation of *A. variabilis* under a diurnal light-dark regime (83). The protective system appears to be regulated independently of nitrogenase synthesis and heterocyst differentiation (304).

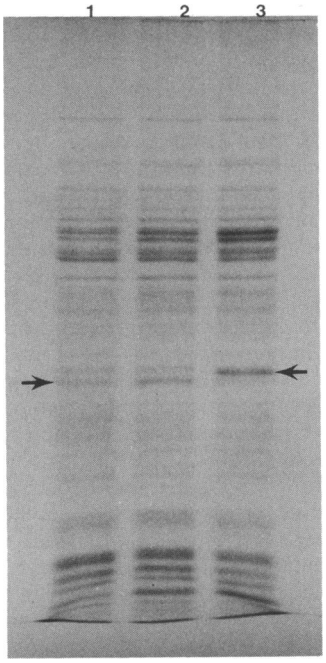

FIG. 9. Electrophoretic analysis of crude extracts of heterocysts isolated from *Anabaena* sp. strain CA grown under N_2-CO_2 (99:1, vol/vol) (lane 1), exposed for 3 h to O_2-CO_2 (99:1, vol/vol) (lane 2), and exposed to O_2-CO_2 (99:1, vol/vol) with chloramphenicol added just before O_2 treatment (lane 3). The arrow indicates the protein modified. Reprinted with permission from Smith et al. (303).

Oxygen stress and protection of nitrogenase in nature. Even though N_2 fixation in heterocyst-forming cyanobacteria is dependent largely on energy and reducing power generated in light reactions, the nitrogenase reaction per se is independent of light (57, 58). Several strains possess the ability to utilize organic substrates (mainly sugars) by heterotrophic metabolism, enabling them to grow and to fix N_2, albeit slowly, in the dark (4, 85, 180). Even in obligate photoautotrophs, such as *A. cylindrica*, N_2 fixation will continue in the dark until endogenous carbon reserves, produced during the previous period of photosynthesis, are available (28, 58, 88). The rate and duration of N_2 fixation in the dark depend on the quantity of incident light absorbed during the light period (88, 160). In planktonic populations of heterocystous cyanobacteria, up to 30% of the total daily amount of N_2 fixed may be fixed in the dark (243) and the nocturnal rates of N_2 fixation may reach 25% of the daytime maximum (327). In general, however, rates of nitrogenase activity in the dark are much below the rates recorded in the light, even in species distinguished by their heterotrophic potential. This situation may stem from an inherent inadequacy of metabolism in the dark to provide sufficient energy and/or reductant to support maximum N_2 fixation (28, 85, 88).

In natural waters, planktonic populations of diazotrophic cyanobacteria are exposed to a complex and rapidly changing environment that is regulated primarily by the daily variations of incident solar radiation. Changes in light quantity alter the rates of photosynthesis and may give rise to considerable fluctuations in the concentrations of dissolved O_2 and inorganic carbon. Under clear-sky conditions, solar radiation increases gradually in the morning hours, reaches a peak at midday, and then declines steadily to nocturnal darkness. Midday photon fluence rates in the surface waters could be inhibitory to photosynthesis and may damage cell structure and cause photooxidative death (1). Cyanobacteria can respond to such conditions by decreasing their pigment concentration, and more particularly by undergoing a relative increase in the concentration of carotenoids, which are known to protect the cells against photooxidation (178, 371). High irradiance, in combination with low concentrations of inorganic carbon and supersaturating levels of dissolved O_2, promotes photorespiration. This is a light-stimulated oxidation of ribulose 1,5-bisphosphate, catalyzed by ribulose-1,5-bisphosphate carboxylase acting as an oxygenase. The reaction may not affect nitrogenase activity in the short term, but could be a drain on the cellular resources of reductant (115, 198).

Under calm conditions, photosynthetic O_2 evolution by phytoplankton can rapidly raise the concentration of dissolved O_2, which may reach supersaturation levels (up to 150 to 200%) by midday (238, 243, 256). Photosynthesis has been shown to be more sensitive to O_2 supersaturation than N_2 fixation is, and it may become inhibited before the nitrogenase activity is seriously affected (243, 256). Rates of nitrogenase activity may decrease initially and recover later

despite continued O_2 supersaturation, while CO_2 fixation remains depressed (178, 293). Planktonic cyanobacteria are able to regulate their buoyancy by the formation or collapse of gas vesicles, which will change their vertical position in the water column (351). Migration to deeper, less illuminated waters as the irradiance increases will help to avoid photoinhibition and photooxidation (256, 268). Rates of N_2 fixation could also be higher where O_2 concentrations are lower, without reserve carbon being depleted to protect nitrogenase (115).

Diurnal variations of photosynthesis and N_2 fixation in planktonic populations of heterocyst-forming cyanobacteria have been measured in a variety of freshwater lakes (115, 256, 322, 327). From these measurements it is evident that the natural situation is extremely complex and is under the influence of a large number of physical, chemical, and biological factors. However, despite differences in the occurrence and relative magnitude of peak values of these two metabolic activities, the patterns of diurnal variation conform with respect to two important aspects: (i) both CO_2 fixation and N_2 fixation are affected by O_2 stress, and (ii) depression of nitrogenase is preceded by the inhibition of photosynthesis. It has been proposed that the temporal separation of their peak activities offers two important advantages to heterocystous cyanobacteria: it enables the buildup of endogenous carbon reserves in heterocysts, and it lessens the competition for reducing power between photosynthesis and N_2 fixation (178, 243).

It is clear that the observed segregation of peak photosynthetic and nitrogenase activities is of little, if any, importance for the protection of nitrogenase from O_2 injury and that heterocysts are eminently equipped with a variety of interrelated mechanisms to prevent the inactivation and destruction of their nitrogenase, even under conditions of extreme O_2 stress.

OXYGEN TENSION AND EXPRESSION OF *nif* GENES IN CYANOBACTERIA

Studies with *Klebsiella*, *Azotobacter*, and *Rhizobium* spp. have shown that oxygen stress causes not only the inactivation of nitrogenase but also the repression of nitrogenase synthesis (276). It appears that the product of the *nifL* gene prevents expression of the *nifHDK* operon under oxygen stress conditions. The precise mechanism of O_2 repression, however, remains largely unknown.

Research during the past 10 years has confirmed that genes encoding the protein components of nitrogenase in cyanobacteria are essentially homologous with corresponding genes present in other N_2-fixing bacteria. However, their organization and expression within the heterocyst-forming genera exhibit some unusual features.

In nonheterocystous cyanobacteria with a potential for N_2 fixation, such as *Gloeothece* (176), *Cyanothece* (174), *Synechococcus* (174), *Plectonema* (8), and *Pseudanabaena* (174, 298) spp., the nitrogenase structural genes, *nifH*, *nifD*, and *nifK*, are organized into a single contiguous *nifHDK* operon within the chromosome, in a similar way to that found in *Klebsiella*, *Azotobacter*, and *Rhizobium* spp. (146). The ability of *Gloeothece* spp. to fix N_2 in air and their mechanism to protect nitrogenase from O_2 injury are apparently regulated by factors (so far unknown) other than the structural genes.

Studies first and foremost by Haselkorn and colleagues have shown that the organization of *nif* genes in heterocystous cyanobacteria, such as in *Anabaena* (269), *Nostoc* (175), and *Calothrix* (175) spp., differs from the above general pattern. In the DNA of vegetative cells, *nifH* is adjacent to *nifD*, but *nifK* is separated from *nifD* by an 11-kbp DNA sequence, designated the *nifD* element (128). The separation of *nifK* and *nifD* genes, coding for the two subunits of dinitrogenase, was surprising since their products are required in equal amounts for an active nitrogenase and are cotranscribed in *Klebsiella* spp. (126). The 11-kbp DNA element, however, is excised from the chromosome of developing heterocysts of *Anabaena* sp. strain PCC7120 during an advanced stage of differentiation, which results in a contiguous *nifHDK* operon and enables simultaneous expression of all three structural *nif* genes (128). The *nifD* element contains the gene *xisA*, which produces a protein, suggested to be a recombinase, responsible for the excision of the *nifD* element (32). The *xisA* gene is essential for N_2 fixation; should it become inactivated, the excision is prevented, no functional nitrogenase is produced, and the organism is unable to grow without a supply of combined nitrogen (129). The *xisA* gene is possibly regulated by a developmental factor that registers the completion of heterocyst differentiation. A similar rearrangement of *nif* structural genes takes place during heterocyst formation in a *Nostoc* species (62).

A second rearrangement results in the deletion of a larger (55-kbp) sequence of DNA, adjacent to the *nifS* gene. The product of this gene is responsible for the maturation of the nitrogenase enzyme complex (127). Both the *nifD* and *nifS* rearrangements occur at a late stage of heterocyst development when the morphological transformation is apparent and concurrent with the synthesis of *nif* mRNA (126). A second *nif* operon, located in the DNA upstream of the *nifHDK* operon, has been identified in *Anabaena* sp. strain PCC7120 (219). It consists of four genes, *nifB*, *fdxN*, *nifS*, and *nifU*, which are probably transcribed as a single operon and only after the excision of the 55-kbp element. *fdxN* codes for a bacterial-type ferredoxin. The product of *nifB* may be required for the synthesis of the Fe-Mo-cofactor. Nothing is yet known of the function of the *nifU* and *nifS* products.

The peculiar organization and rearrangement of *nif* genes in heterocystous cyanobacteria appear to function by restricting *nif* gene expression to fully developed heterocysts, which are equipped with all the structural and biochemical properties that protect nitrogenase from the effects of oxygen stress. Results of a study by Elhai and Wolk (79), in which the transcription of *nif* structural genes was assayed by means of light emission following the fusion of the *nif* gene promoter to the luciferase genes, strongly support the view that the induction of *nif* genes is regulated by a developmental, and not environmental, signal(s) associated with the process of heterocyst differentiation. The observations also indicate that regulation of gene expression occurs at the transcriptional level. The conclusions gained further support from studies with mutants of *Anabaena* sp. strain PCC7120 defective in the heterocyst envelope (192). The apparently higher intracellular O_2 tensions did not prevent DNA rearrangement in the mutant strains. A number of experimental data, however, argue for a rather complex system of regulation of *nif* gene expression in heterocyst-forming cyanobacteria. When cultures of *Anabaena* sp. strain PCC7120 were incubated under argon (in the absence of O_2, N_2, and CO_2), following transfer to a medium free from combined nitrogen, only the 55-kbp DNA excision occurred and the cultures displayed neither morphological differentiation nor nitrogenase activity (127). In another study, in which *A. variabilis* was incubated under argon in

the presence of DCMU, nitrogenase mRNA was synthesized and nitrogenase activity was detected after a short period of nitrogen starvation, but heterocyst differentiation was suppressed. When such filaments were subsequently exposed to O_2, nitrogenase mRNA levels declined rapidly (151). In a heterocystless (*het*) mutant of *Nostoc* sp. strain PCC7906, rearrangement of *nif* structural genes was found to occur in all cells, but only in the presence of O_2 (62). The expression of the *nifHDK* promoter is possibly controlled by O_2, but its repression seems to require a pO_2 greater than that at which nitrogenase is inactivated (80). These observations appear to imply a possible role for O_2 in the process of DNA rearrangement, but so far little is known of the molecular mechanism by which levels of O_2 are sensed and gene expression is regulated in cyanobacteria in response to oxygen tensions.

Surprisingly, DNA in a strain of the heterocystous genus *Fischerella* has a contiguous arrangement of *nif* structural genes, similar to that present in nonheterocystous cyanobacteria (283). *Fischerella* belongs to the order *Stigonematales*, which comprises forms with different patterns of ultrastructure, heterocyst spacing, and heterocyst development, compared with that observed in members of the order *Nostocales* (230). It has been suggested that members of the order *Stigonematales* represent a more primitive stage in cyanobacterial evolution, possibly linking the coccoid and filamentous forms (206).

EVOLUTIONARY CONSIDERATIONS

Because of the paucity and inconclusive nature of relevant geological and fossil records, our knowledge of the primeval environment and of the earliest forms of life is based mostly on geophysical and geochemical considerations (20, 205, 287). According to a widely accepted hypothesis, the primordial atmosphere most probably had a reducing nature and changed later to a redox-neutral state before becoming aerobic (31). It is generally believed that the first living organisms were heterotrophic anaerobes and that they were dependent on geochemically produced organic matter for their metabolism. It has also been presumed that nitrogen was present in abundant quantity in its most reduced form, ammonia, which was utilized by the ancient microorganisms for the biosynthesis of cell material.

Since nitrogenase synthesis is repressed by both ammonia and oxygen, it seems reasonable to assume that the evolution of nitrogen fixation was preceded by the exhaustion or limited availability of combined nitrogen, the presence of free N_2, and the absence (or near absence) of free O_2 in the contemporary environment. The ability to fix N_2 was probably acquired first by ancient fermenters, similar to the present-day clostridia. N_2-fixing potential, even at present, is more widely distributed among anaerobic and facultative bacteria than aerobic bacteria (261). From similar considerations, the evolution of chemo- and photoautotrophy was probably a consequence of the exhaustion of organic nutrients in the ancient oceans.

There is convincing indirect evidence to support the view that about 2 billion years ago, the primitive environment was virtually anoxic (50, 159). Before the evolution of oxygenic photosynthesis, the O_2 content of the atmosphere was only about 0.2%, and many, if not most, ancient cyanobacteria probably inherited and carried genetic information for the synthesis of nitrogenase. The evolution of O_2-producing photosynthesis in cyanobacteria was undoubtedly the most important event of the Precambrian period and determined, more than any other circumstance, the further course of biological evolution. Atmospheric O_2 concentrations seem to have changed considerably during the following 1.5 billion years and have remained fairly steady only during the last 350 million years of Earth history. The gradual buildup of atmospheric O_2 content, as a result of the proliferation of cyanobacteria in the Precambrian period (287), had created conditions for the evolution of aerobic respiration and for the diversification of metabolic systems and life-forms. On the other hand, the increasingly oxic environment has confined N_2-fixing organisms to anaerobic habitats. As a result of selective evolutionary pressures, a number of microaerobic and aerobic bacteria, including cyanobacteria, have apparently acquired biochemical and structural properties enabling them to protect their nitrogenase from inactivation or destruction by oxygen and to fix N_2 under conditions of elevated O_2 tension.

Microfossil records from the early Precambrian period are rare because of the paucity of nonmetamorphosed sedimentary rocks, and they are also indistinct because of the lack of hard, readily preservable structures in the ancient microorganisms. Earlier reports on presumptive cyanobacterial microfossils from rocks about 3.5 to 3.8 billion years old are now considered to be of dubious authenticity and provide inadequate evidence for the existence of cyanobacteria (287). Nonetheless, Schopf and Packer (286) have more recently reported on early Archean microfossils from carbonaceous cherts about 3.3 to 3.5 billion years old, which apparently preserved a mixed population of colonial and filamentous forms, including forms "suggestive" of chroococcalean cyanobacteria. More convincing evidence of fossil cyanobacteria has been obtained from stromatolites (laminated sedimentary rocks) about 2.5 to 2.8 billion years old, which contain fossil remains of ancient mat-forming communities, including filamentous structures resembling modern oscillatoriacean cyanobacteria (287).

In view of the rarity and ambiguity of fossil records, molecular approaches to the study of bacterial evolution have gained increasing application and recognition. Molecular methods for the assessment of genetic relatedness include, among others, the comparative analysis and amino acid sequencing of proteins, the nucleotide sequencing of DNA and RNA, analysis of DNA base composition, and determination of genome size and DNA hybridization. The comparative analysis of oligonucleotide sequences of 16S rRNAs has become one of the most reliable and expedient methods of determining prokaryotic genealogies. The principal reason for this is that rRNAs are universally distributed and seem to change very little, thus retaining their structure and function throughout phylogeny. This methodology has been extensively and successfully applied in recent years to establish the evolutionary relationships among prokaryotes.

From the results of an extensive analytical survey by Fox et al. (103), it was possible to draw a number of important conclusions. First, the most ancient bacterial phenotypes were found to be anaerobic; aerobic phenotypes have arisen a number of times during bacterial evolution. Second, many nonphotosynthetic bacteria appear to have evolved from photosynthetic ancestors, a finding that casts doubt on the belief that the first bacteria were heterotrophic. Third, unicellular cyanobacteria seemed to represent a diverse grouping of the most ancient cyanobacterial forms that gave rise to more advanced forms of cyanobacteria.

A more recent study by Giovannoni et al. (123) provided further details of the evolutionary relationships among cyanobacteria. Their results imply that (i) the major eubacterial

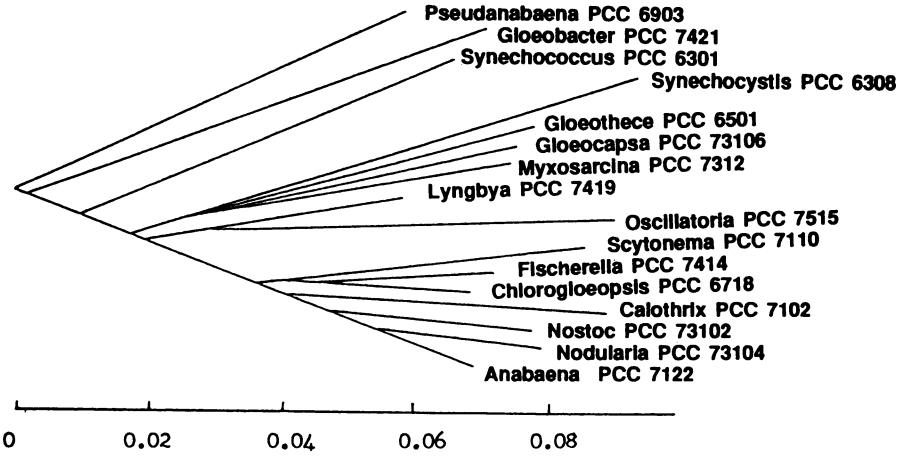

FIG. 10. Cyanobacterial evolutionary relationships based on comparative analysis of 16S rRNA sequences. Reprinted with permission from Giovannoni et al. (123).

lines, including anaerobic phototrophic bacteria, diverged verifiably before the diversification of cyanobacteria; (ii) the divergence of cyanobacterial lines occurred within a relatively short evolutionary time; and (iii) heterocystous cyanobacteria constitute a distinct phylogenetic assemblage that has evolved significantly later than other cyanobacterial lines (Fig. 10).

It is plain from the foregoing findings and considerations that much more information is required to substantiate preliminary conclusions about the progression of cyanobacterial evolution from the simple unicellular forms to colonial, filamentous, nonheterocystous, and heterocystous structures. Nevertheless, the indications so far available support the view that the gradual buildup of atmospheric O_2 concentrations was paralleled by the development of more complex structural and physiological properties in ancient cyanobacteria. Evidence is mounting that this progression also involved the acquisition of structures and mechanisms for the efficient protection and function of the N_2-fixing enzyme system in cyanobacteria.

CONCLUDING REMARKS

Geological records and 16S mRNA-based phylogenetic analyses suggest that N_2 fixation preceded oxygenic photosynthesis during cyanobacterial evolution. Accordingly, the primary necessity of protecting nitrogenase from O_2 released within the same cell must have arisen in ancient cyanobacteria concurrently with the evolution of oxygenic photosynthesis and long before atmospheric O_2 concentrations reached inhibitory levels. We may presume that some structural and/or biochemical mechanisms capable of negating the intracellular O_2 stress developed during this early period. With the gradual buildup of the O_2 concentration in the atmosphere, an additional need arose to protect nitrogenase from the external O_2 diffusing into N_2-fixing cells. Some diazotrophic cyanobacteria were able to acquire supplementary and more efficient mechanisms to protect their nitrogenase. Others, being unable to adapt, could fix N_2 only in oxygen-free habitats or under microaerobic conditions. Research during the past two decades has discovered a wide range of oxygen tolerance among diazotrophic cyanobacteria and great diversity in their response to O_2 stress. It is tempting to speculate that this variety reflects stages in the adaptation of cyanobacteria to O_2 enrichment of their environment.

Following progress made earlier in the fields of physiology and ecology of N_2 fixation in cyanobacteria, our knowledge of the biochemical regulation and genetic control of cyanobacterial N_2 fixation has advanced considerably during the past 10 years. Nevertheless, our understanding of the protection of nitrogenase from the damaging effects of endogenous and exogenous O_2 in non-heterocystous cyanobacteria has progressed relatively slowly. Many questions remain to be answered (such as the cellular location of nitrogenase activity in relation to the sites of photosynthetic and respiratory electron transport systems, the supply of reductant and ATP in the light and the dark, and the existence of spatial and/or temporal separation of photosynthesis and nitrogenase activity within a single cell or a single trichome) before a convincing interpretation could be presented of how nonheterocystous cyanobacteria are able to maintain a functional nitrogenase in O_2-evolving cells. Recent reports on the existence of conformational protection, associated with the reversible modification of dinitrogenase reductase, are revealing, but our knowledge of other potential mechanisms, such as oxyhydrogen reaction, enzymatic protection, and light-dependent O_2 consumption or photorespiration, is inadequate and will no doubt be pursued further.

A number of heterocystous cyanobacteria develop in symbiotic associations with plants of diverse structural and physiological complexity. In these associations the cyanobionts are fixing N_2 at much higher rates than in isolation, and most of the fixed nitrogen is taken over by the host organism (324). N_2 fixation in the cyanobiont appears to be stimulated in the microaerobic environment maintained by the host organism and by the efficient transfer of fixed nitrogen. In view of recent advances in bioengineering, the prospects of artificially creating stable cyanobacterium-crop plant associations need no longer be an illusion (116). Such associations could be of great benefit to crop plants and could reduce the demand for chemical fertilizers.

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